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(71) Applicant: COLD SPRING HARBOR LABORATORY [US/US]; 100 Bungtown Road, Cold Spring Harbor, NY 11724 (US).

(72) Inventor: BEACH, David, H. ; 19 Woodland Drive, Huntington Bay, NY 11743 (US).

(74) Agents: GRANAHAN, Patricia et al.; Hamilton, Brook, Smith & Reynolds, Two Militia Drive, Lexington, MA 02173 (US).

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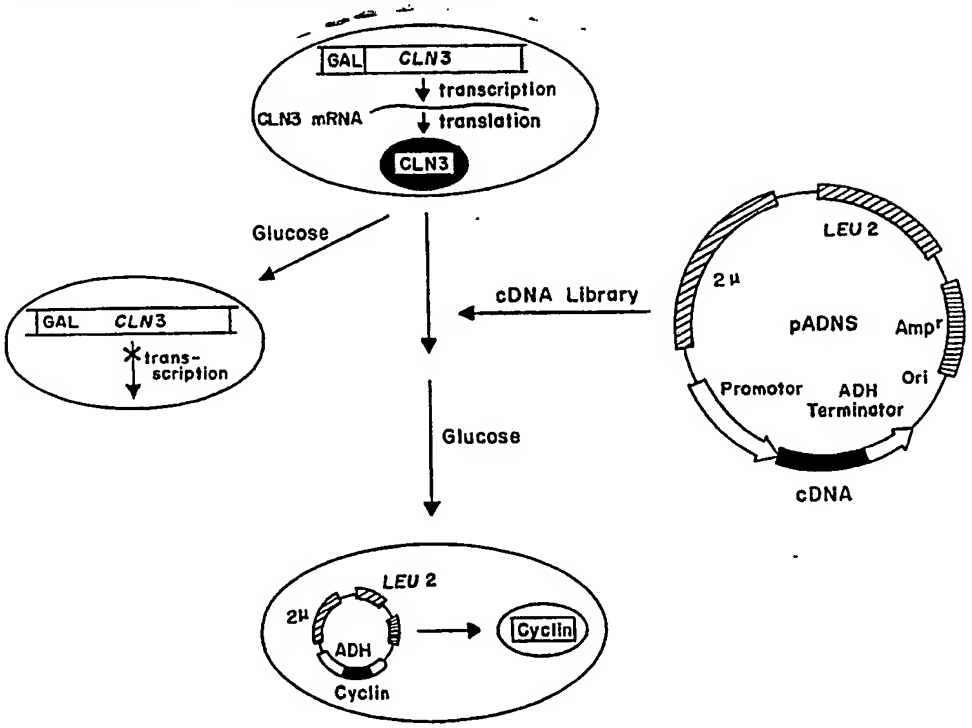

(57) Abstract

A novel class of cyclins, referred to as D-type cyclins, of mammalian origin, particularly human origin, DNA and RNA encoding the novel cyclins, and a method of identifying other D-type and non-D-type cyclins. Also disclosed are a method of detecting an increased level of a D-type cyclin and a method of inhibiting cell division by interfering with formation of the protein kinase-D-type cyclin complex essential for cell cycle start.

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D-TYPE CYCLIN AND USES RELATED THERETO

Description

Background of the Invention

A typical cell cycle of a eukaryotic cell includes the M phase, which includes nuclear division (mitosis) and cytoplasmic division or cytokinesis and interphase, which begins with the G1 phase, proceeds into the S phase and ends with the G2 phase, which continues until mitosis begins, initiating the next M phase. In the S phase, DNA replication and histone synthesis occurs, while in the G1 and G2 phases, no net DNA synthesis occurs, although damaged DNA can be repaired. There are several key changes which occur during the cell cycle, including a critical point in the G1 phase called the restriction point or start, beyond which a cell is committed to completing the S, G2 and M phases.

Onset of the M phase appears to be regulated by a common mechanism in all eukaryotic cells. A key element of this mechanism is the protein kinase $p34^{cdc2}$, whose activation requires changes in phosphorylation and interaction with proteins referred to as cyclins, which also have an ongoing role in the M phase after activation.

Cyclins are proteins that were discovered due to their intense synthesis following the fertilization of marine invertebrate eggs (Rosenthal, E.T. et al., Cell 20:487-494 (1980)). It was subsequently observed that the abundance of two types of cyclin, A and B, oscillated

during the early cleavage divisions due to abrupt proteolytic degradation of the polypeptides at mitosis and thus, they derived their name (Evans, T. et al., Cell 33:389-396 (1983); Swenson, K.I. et al., Cell 47:867-870 (1986); Standart, N. et al., Dev. Biol. 124:248-258 (1987)).

Active rather than passive involvement of cyclins in regulation of cell division became apparent with the observation that a clam cyclin mRNA could cause activation of frog oocytes and entry of these cells into M phase (Swenson, K.I. et al., Cell 47:867-870 (1986)). Activation of frog oocytes is associated with elaboration of an M phase inducing factor known as MPF (Masui, Y. and C.L. Markert, J. Exp. Zool. 177:129-146 (1971); Smith, L.D. and R.E. Ecker, Dev. Biol. 25:232-247 (1971)). MPF is a protein kinase in which the catalytic subunit is the frog homolog of the cdc2 protein kinase (Dunphy, W.G. et al., Cell 54:423-431 (1988); Gautier, J. et al., Cell 54:433-439 (1988); Arion, D. et al., Cell 55:371-378 (1988)).

Three types of classes of cyclins have been identified to date: B, A and CLN cyclins. The B-type cyclin has been shown to act in mitosis by serving as an integral subunit of the cdc2 protein kinase (Booher, R. and D. Beach, EMBO J. 6:3441-3447 (1987); Draetta, G. et al., Cell 56:829-838 (1989); Labbe, J.C. et al., Cell 57:253-263 (1989); Labbe, J.C. et al., EMBO J. 8:3053-3058 (1989); Meijer, L. et al., EMBO J. 8:2275-2282 (1989); Gautier, J. et al., Cell 60:487-494 (1990)). The A-type cyclin also independently associates with the cdc2

kinase, forming an enzyme that appears to act earlier in the division cycle than mitosis (Draetta, G. et al., Cell 56:829-838 (1989); Minshull, J. et al., EMBO J. 9:2865-2875 (1990); Giordano, A. et al., Cell 58:981-990 (1989); Pines, J. and T. Hunter, Nature 346:760-763 (1990)). The functional difference between these two classes of cyclins is not yet fully understood.

Cellular and molecular studies of cyclins in invertebrate and vertebrate embryos have been accompanied by genetic studies, particularly in ascomycete yeasts. In the fission yeast, the cdc13 gene encodes a B-type cyclin that acts in cooperation with cdc2 to regulate entry into mitosis (Booher, R. and D. Beach, EMBO J., 6:3441-3447 (1987); Booher, R. and D. Beach, EMBO J. 7:2321-2327 (1988); Hagan, I. et al., J. Cell Sci. 91:587-595 (1988); Solomon, M., Cell 54:738-740 (1988); Goebl, M. and B. Byers, Cell 54:433-439 (1988); Booher, R.N. et al., Cell 58:485-497 (1989)).

Genetic studies in both the budding yeast and fission yeast have revealed that cdc2 (or CDC28 in budding yeast) acts at two independent points in the cell cycle: mitosis and the so-called cell cycle "start" (Hartwell, L.H., J. Mol. Biol., 104:803-817 (1971); Nurse, P. and Y. Bissett, Nature 292:558-560 (1981); Piggot, J.R. et al., Nature 298:391-393 (1982); Reed, S.I. and C. Wittenberg, Proc. Nat. Acad. Sci. USA 87:5697-5701 (1990)).

In budding yeast, the start function of the CDC28 protein also requires association of the catalytic subunit of the protein kinase with ancillary proteins

that are structurally related to A and B-type cyclins. This third class of cyclin has been called the Cln class, and three genes comprising a partially redundant gene family have been described (Nash, R. et al., EMBO J. 7:4335-4346 (1988); Hadwiger, J.A. et al., Prot. Natl. Acad. Sci. USA 86:6255-6259 (1989); Richardson, H.E. et al., Cell 59:1127-1133 (1989)). The CLN genes are essential for execution of start and in their absence, cells become arrested in the G1 phase of the cell cycle. The CLN1 and CLN2 transcripts oscillate in abundance through the cell cycle, but the CLN3 transcript does not. In addition, the Cln2 protein has been shown to oscillate in parallel with its mRNA (Nash, R. et al., EMBO J. 7:4335-4346 (1988); Cross, F.R., Mol. Cell. Biol. 8:4675-4684 (1988); Richardson, H.E. et al., Cell 59:1127-1133 (1988); Wittenberg, et al., 1990)).

Although the precise biochemical properties conferred on cdc2/CDC28 by association with different cyclins have not been fully elaborated, genetic studies of cyclin mutants clearly establishes that they confer "G1" and "G2" properties on the catalytic subunit (Booher, R. and D. Beach, EMBO J. 6:3441-3447 (1987); Nash, R. et al., EMBO J. 7:4335-4346 (1988); Richardson, H.E. et al., Cell 56:1127-1133 (1989)).

cdc2 and cyclins have been found not only in embryos and yeasts, but also in somatic human cells. The function of the cdc2/cyclin B enzyme appears to be the same in human cells as in other cell types (Riabowol, K. et al., Cell 57:393-401 (1989)). A human A type cyclin has also been found in association with cdc2. No CLN

type cyclin has yet been described in mammalian cells. A better understanding of the elements involved in cell cycle regulation and of their interactions would contribute to a better understancing of cell replication and perhaps even alter or control the process.

Summary of the Invention

The present invention relates to a novel class of cyclins, referred to as D-type cyclins, which are of mammalian origin and are a new family of cyclins related to, but distinct from, previously described A, B or CLN type cyclins. In particular, it relates to human cyclins, encoded by genes shown to be able to replace a CLN-type gene essential for cell cycle start in yeast, which complement a deficiency of a protein essential for cell cycle start and which, on the basis of protein structure, are on a different branch of the evolutionary tree from A, B or CLN type cyclins. Three members of the new family of D-type cyclins, referred to as the human D-type gene family, are described herein. They encode small (33-34 KDa) proteins which share an average of 57% identity over the entire coding region and 78% in the cyclin box. One member of this new cyclin family, cyclin D1 or CCND1, is 295 amino acid residues and has an estimated molecular weight of 33,670 daltons (Da). A second member, cyclin D2 or CCND2, is 289 amino acid residues and has an estimated molecular weight of 33,045 daltons. It has been mapped to chromosome 12p band p13. A third member, cyclin D3 or CCND3, is 292 amino acid residues and has an estimated molecular weight of approximately 32,482 daltons. It has been mapped to

chromosome 6p band p21. The D-type cyclins described herein are the smallest cyclin proteins identified to date. All three cyclin genes described herein are interrupted by an intron at the same position. D-type cyclins of the present invention can be produced using recombinant techniques, can be synthesized chemically or can be isolated or purified from sources in which they occur naturally. Thus, the present invention includes recombinant D-type cyclins, isolated or purified D-type cyclins and synthetic D-type cyclins.

The present invention also relates to DNA or RNA encoding a D-type cyclin of mammalian origin, particularly of human origin, as well as to antibodies, both polyclonal and monoclonal, specific for a D-type cyclin of mammalian, particularly human, origin.

The present invention further relates to a method of isolating genes encoding other cyclins, such as other D-type cyclins and related (but non-D type) cyclins. It also has diagnostic and therapeutic aspects. For example, it relates to a method in which the presence and/or quantity of a D-type cyclin (or cyclins) in tissues or biological samples, such as blood, urine, feces, mucous or saliva, is determined, using a nucleic acid probe based on a D-type cyclin gene or genes described herein or an antibody specific for a D-type cyclin. This embodiment can be used to predict whether cells are likely to undergo cell division at an abnormally high rate (i.e., if cells are likely to be cancerous), by determining whether their cyclin levels or activity are elevated (elevated level of activity being indicative of an increased probability that cells will

undergo an abnormally high rate of division). The present method also relates to a diagnostic method in which the occurrence of cell division at an abnormally high rate is assessed based on abnormally high levels of a D-type cyclin(s), a gene(s) encoding a D-type cyclin(s) or a transcription product(s) (RNA).

In addition, the present invention relates to a method of modulating (decreasing or enhancing) cell division by altering the activity of at least one D-type cyclin, such as D2, D2 or D3 in cells. The present invention particularly relates to a method of inhibiting increased cell division by interfering with the activity or function of a D-type cyclin(s). In this therapeutic method, function of D-type cyclin(s) is blocked (totally or partially) by interfering with its ability to activate the protein kinase it would otherwise (normally) activate (e.g., $p34^{cdc2}$ or a related protein kinase), by means of agents which interfere with D-type cyclin activity, either directly or indirectly. Such agents include anti-sense sequences or other transcriptional modulators which bind D cyclin-encoding DNA or RNA; antibodies which bind either the D-type cyclin or a molecule with which a D-type cyclin must interact or bind in order to carry out its role in cell cycle start; substances which bind the D-type cyclin(s); agents (e.g., proteases) which degrade or otherwise inactivate the D-type cyclin(s); or agents (e.g., small organic molecules) which interfere with association of the D-type cyclin with the catalytic subunit of the kinase. The subject invention also relates to agents (e.g., oligonucleotides, antibodies, peptides) useful in the isolation, diagnostic or therapeutic methods described.

Brief Description of the Figures

Figure 1 is a schematic representation of a genetic screen for human cyclin genes.

Figure 2 is the human cyclin D1 nucleic acid sequence (SEQ ID No. 1) and amino acid sequence (SEQ ID No. 2), in which nucleotide numbers and amino acid numbers are on the right, amino acid numbers are given with the initiation methionine as number one and the stop codon is indicated by an asterisk.

Figure 3 is the human cyclin D2 nucleic acid sequence (SEQ ID No. 3) and amino acid sequence (SEQ ID No. 4) in which nucleotide numbers and amino acid numbers are on the right, amino acid numbers are given with the initiation methionine as number one and the stop codon is indicated by an asterisk.

Figure 4 is the human cyclin D3 nucleic acid sequence (SEQ ID No. 5) and amino acid sequence (SEQ ID No. 6), in which nucleotide numbers and amino acid numbers are on the right, amino acid numbers are given with the initiation methionine as number one and the stop codon is indicated by an asterisk.

Figure 5 shows the cyclin gene family.

Figure 5A shows the amino acid sequence alignment of seven cyclin genes (CYCD1-Hs, SEQ ID No. 7; CYCA-Hs, SEQ ID No. 8; CYCA-Dm, SEQ ID No. 9; CYCB1-Hs, SEQ ID No. 10; CDC13-Sp, SEQ ID No. 11; CLN1-Sc, SEQ ID No. 12; CLN3-Sc, SEQ ID No. 13), in which numbers within certain sequences indicate the number of amino acid residues omitted from the sequence as the result of insertion.

Figure 5B is a schematic representation of the evolutionary tree of the cyclin family, constructed using the Neighbor-Joining method; the length of horizontal line reflects the divergence.

Figure 6 shows alternative polyadenylation of the cyclin D1 gene transcript.

Figure 6A is a comparison of several cDNA clones isolated from different cell lines. Open boxes represent the 1.7 kb small transcript containing the coding region of cyclin D1 gene. Shadowed boxes represent the 3' fragment present in the 4.8 kb long transcript. Restriction sites are given above each cDNA clone to indicate the alignment of these clones.

Figure 6B shows the nucleotide sequence surrounding the first polyadenylation site for several cDNA clones (CYCD1-21, SEQ ID No. 14; CYCD1-H12, SEQ ID No. 15; CYCD1-HO34, SEQ ID No. 16; CYCD1-TO78, SEQ ID No. 17 and a genomic clone; CYCD1-GO68, SEQ ID No. 18).

Figure 6C is a summary of the structure and alternative polyadenylation of the cyclin D1 gene. Open boxes represent the small transcript, the shadowed box represents the 3' sequence in the large transcript and the filled boxes indicate the coding regions.

Figure 7 shows the protein sequence comparison of eleven mammalian cyclins (CYCD1-Hs, SEQ ID No. 19; CYL1-Mm, SEQ ID No. 20; CYCD2-Hs, SEQ ID No. 21; CYCL2-Mm, SEQ ID No. 22; CYCD3-Hs, SEQ ID No. 23; CYL3-Mm, SEQ ID No. 24; CYCA-Hs, SEQ ID No. 25; CYCB1-Hs, SEQ ID No. 26; CYCB2-Hs, SEQ ID No. 27; CYCC-Hs, SEQ ID No. 28; CYCE-Hs, SEQ ID No. 29).

Figure 8 is a schematic representation of the genomic structure of human cyclin D genes, in which each diagram represents one restriction fragment from each cyclin D gene that has been completely sequenced. Solid

boxes indicate exon sequences, open boxes indicate intron or 5′ and 3′ untranslated sequences and hatched boxes represent pseudogenes. The positions of certain restriction sites, ATG and stop codons are indicated at the top of each clone.

Figure 9 is the nucleic acid sequence (SEQ ID No. 30) and amino acid sequence (SEQ ID No. 31) of a cyclin D2 pseudogene.

Figure 10 is the nucleic acid sequence (SEQ ID No. 32) and the amino acid sequence (SEQ ID No. 33) of a cyclin D3 pseudogene.

Figure 11 is the nucleic acid sequence (SEQ ID No. 34) of 1.3 kb of human cyclin D1 promoter; the sequence ends at initiation ATG codon and transcription starts at approximately nucleotide -160.

Figure 12 is the nucleotide sequence (SEQ ID No. 35) of 1.6 kb of human cyclin D2 promoter; the sequence ends at initiation ATG codon and transcription starts at approximately nucleotide -170.

Figure 13 is the nucleotide sequence (SEQ ID No. 36) of 3.2 kb of human cyclin D3 promoter; the sequence ends at initiation ATG codon and transcription starts at approximately nucleotide -160.

Detailed Description of the Invention

As described herein, a new class of mammalian cyclin proteins, designated D-type cyclins, has been identified, isolated and shown to serve as a control element for the cell cycle start, in that they fill the role of a known cyclin protein by activating a protein kinase whose activation is essential for cell cycle start, an event in

the G1 phase at which a cell becomes committed to cell division. Specifically, human D-type cyclin proteins, as well as the genes which encode them, have been identified, isolated and shown to be able to replace CLN type cyclin known to be essential for cell cycle start in yeast. The chromosomal locations of CCND2 and CCND3 have also been mapped.

As a result, a new class of cyclins (D type) is available, as are DNA and RNA encoding the novel D-type cyclins, antibodies specific for (which bind to) D-type cyclins and methods of their use in the identification of additional cyclins, the detection of such proteins and oligonucleotides in biological samples, the inhibition of abnormally increased rates of cell division and the identification of inhibitors of cyclins.

The following is a description of the identification and characterization of human D-type cyclins and of the uses of these novel cyclins and related products.

Isolation and Characterization of Human Cyclin D1, D2 and D3

As represented schematically in Figure 1 and described in detail in Example 1, a mutant yeast strain in which two of the three CLN genes (CLN1 and CLN2) were inactive and expression of the third was conditional, was used to identify human cDNA clones which rescue yeast from CLN deficiency. A human glioblastoma cDNA library carried in a yeast expression vector (pADNS) was introduced into the mutant yeast strain. Two yeast transformants (pCYCD1-21 and pCYCD1-19) which grew despite the

lack of function of all three CLN genes and were not revertants, were identified and recovered in E. coli. Both rescued the mutant (CLN deficient) strain when reintroduced into yeast, although rescue was inefficient and the rescued strain grew relatively poorly.

pCYCD1-19 and pCYCD1-21 were shown, by restriction mapping and partial DNA sequence analysis, to be independent clones representing the same gene. A HeLa cDNA library was screened for a full length cDNA clone, using the 1.2 kb insert of pCYCD1-21 as probe. Complete sequencing was done of the longest of nine positive clones identified in this manner (pCYCD1-H12; 1325 bp). The sequence of the 1.2 kb insert is presented in Figure 2; the predicted protein product of the gene is of approximate molecular weight 34,000 daltons.

Cyclin D2 and cyclin D3 cDNAs were isolated using the polymerase chain reaction and three oligonucleotide probes derived from three highly conserved regions of D-type cyclins, as described in Example 4. As described, two 5' oligonucleotides and one 3' degenerate oligonucleotide were used for this purpose. The nucleotide and amino acid sequences of the CCND2 gene and encoded D2 cyclin protein are represented in Figure 3 and of the CCND3 gene and encoded D3 cyclin protein are represented in Figure 4. A deposit of plasmid pCYC-D3 was made with the American Type Culture Collection (Rockville, MD) on May 14, 1991, under the terms of the Budapest Treaty. Accession number 68620 has been assigned to the deposit.

Comparison of the CYCD1-H12-encoded protein sequence with that of known cyclins (see Figure 5A) showed that

there was homology between the new cyclin and A, B and CLN type cyclins, but also made it clear that CYCD1 differs from these existing classes.

An assessment of how this new cyclin gene and its product might be related in an evolutionary sense to other cyclin genes was carried out by a comprehensive comparison of the amino acid sequences of all known cyclins (Figure 5B and Example 1). Results of this comparison showed that CYCD1 represents a new class of cyclin, designated herein cyclin D.

Expression of cyclin D1 gene in human cells was studied using Northern analysis, as described in Example 2. Results showed that levels of cyclin D1 expression were very low in several cell lines. The entire coding region of the CYCD1 gene was used to probe poly(A)+ RNA from HeLa cells and demonstrated the presence of two major transcripts, one approximately 4.8 kb and the other approximately 1.7 kb, with the higher molecular weight form being the more abundant. Most of the cDNA clones isolated from various cDNA libraries proved to be very similar to clone λCYCD1-H12 and, thus, it appears that the 1.7 kb transcript detected in Northern blots corresponds to the nucleotide sequence of Figure 2. The origin of the larger (4.8 kb) transcript was unclear. As described in Example 2, it appears that the two mRNAs detected (4.8 kb and 1.7 kb) arose by differential polyadenylation of CYCD1 (Figure 6).

Differential expression of cyclin D1 in different tissues and cell lines was also assessed, as described in Example 3. Screening of cDNA libraries to obtain full length CYCD1 clones had demonstrated that the cDNA

library from the human glioblastoma cell line (U118 MG) used to produce yeast transformants produced many more positives than the other three cDNA libraries (human HeLa cell cDNA, human T cell cDNA, human teratocarcinoma cell cDNA). Northern and Western blotting were carried out to determine whether cyclin D1 is differentially expressed. Results showed (Example 3) that the level of transcript is 7 to 10 fold higher in the glioblastoma (U118 MG) cells than in HeLa cells, and that in both HeLa and U118 MG cells, the high and low molecular weight transcripts occurred. Western blotting using anti-CYL1 antibody readily detected the presence of a 34kd polypeptide in the glioblastoma cells and demonstrated that the protein is far less abundant in HeLa cells and not detectable in the 293 cells. The molecular weight of the anti-CYCL1 cross reactive material identified in U118 MG and HeLa cells is exactly that of the human CYCD1 protein expressed in E. coli. Thus, results demonstrated differential occurrence of the cyclin D1 in the cell types analyzed, with the highest levels being in cells of neural origin.

As also described herein (Example 6), human genomic libraries were screened using cDNA probes and genomic clones of human D-type cyclins, specifically D1, D2 and D3, have been isolated and characterized. Nucleic acid sequences of cyclin D1, D2 and D3 promoters are represented in Figures 11-13. Specifically, the entire 1.3 kb cyclin D1 cDNA clone was used as a probe to screen a normal human liver genomic library, resulting in identification of three positive clones. One of these clones (G6) contained a DNA insert shown to contain 1150 bp of

upstream promoter sequence and a 198 bp exon, followed by an intron. Lambda genomic clones corresponding to the human cyclin D2 and lambda genomic clones corresponding to the human cyclin D3 were also isolated and characterized, using a similar approach. One clone (γD2-G4) was shown to contain (Figure 8B) a 2.7 kb SacI SmaI fragment which includes 1620 bp of sequence 5' to the presumptive initiating methionine codon identified in D2 cDNA (Figure 3) and a 195 bp exon followed by a 907 bp intervening sequence. One clone (G9) was shown to contain (Figure 8C) 1.8 kb of sequence 5' to the presumptive initiating methionine codon identified in D3 cDNA (Figure 4), a 198 bp exon 1, a 684 bp exon 2 and a 870 bp intron.

Thus, as a result of the work described herein, a novel class of mammalian cyclins, designated cyclin D or D-type cyclin, has been identified and shown to be distinct, on the basis of structure of the gene (protein) product, from previously-identified cyclins. Three members of this new class, designated cyclin D1 or CCND1, cyclin D2 or CCND2 and cyclin D3 or CCND3, have been isolated and sequenced. They have been shown to fulfill the role of another cyclin (CLN type) in activation of the protein kinase (CDC28) which is essential for cell cycle start in yeast. It has also been shown that the cyclin D1 gene is expressed differentially in different cell types, with expression being highest in cells of neural origin.

Uses of the Invention

It is possible, using the methods and materials described herein, to identify genes (DNA or RNA) which

encode other cyclins (DNA or RNA which replaces a gene essential for cell cycle start). This method can be used to identify additional members of the cyclin D class or other (non-D type) cyclins of either human or nonhuman origin. This can be done, for example, by screening other cDNA libraries using the budding yeast strain conditional for CLN cyclin expression, described in Example 1, or another mutant in which the ability of a gene to replace cyclin expression can be assessed and used to identify cyclin homologues. This method is carried out as described herein, particularly in Example 1 and as represented in Figure 1. A cDNA library carried in an appropriate yeast vector (e.g., pADNS) is introduced into a mutant yeast strain, such as the strain described herein (Example 1 and Experimental Procedures). The strain used contains altered CLN genes. In the case of the specific strain described herein, insertional mutations in the CLN1 and CLN2 genes rendered them inactive and alteration of the CLN3 gene allowed for its conditional expression from a galactose-inducible, glucose-repressible promoter; as exemplified, this promoter is a galactose-inducible, glucose-repressible promoter but others can be used.

Mutant yeast transformed with the cDNA library in the expression vector are screened for their ability to grow on glucose-containing medium. In medium containing galactose, the CLN3 gene is expressed and cell viability is maintained, despite the absence of CLN1 and CLN2. In medium containing glucose, all CLN function is lost and the yeast cells arrest in the G1 phase of the cell cycle. Thus, the ability of a yeast transformant to grow on

glucose-containing medium is an indication of the presence in the transformant of DNA able to replace the function of a gene essential for cell cycle start. Although not required, this can be confirmed by use of an expression vector, such as pADNS, which contains a selectable marker (the LEU2 marker is present in pADNS). Assessment of the plasmid stability shows whether the ability to grow on glucose-containing medium is the result of reversion or the presence of DNA function (introduction of DNA which replaces the unexpressed or nonfunctional yeast gene(s) essential for cell cycle start). Using this method, cyclins of all types (D type, non-D type) can be identified by their ability to replace CLN3 function when transformants are grown on glucose.

Screening of additional cDNA or genomic libraries to identify other cyclin genes can be carried out using all or a portion of the human D-type cyclin DNAs disclosed herein as probes; for example, all or a portion of the D1, D2 or D3 cDNA sequences of Figures 2-4, respectively, or all or a portion of the corresponding genomic sequences described herein can be used as probes. The hybridization conditions can be varied as desired and, as a result, the sequences identified will be of greater or lesser complementarity to the probe sequence (i.e., if higher or lower stringency conditions are used). Additionally, an anti-D type cyclin antibody, such as CYL1 or another raised against D1 or D3 or other human D-type cyclin, can be used to detect other recombinant D-type cyclins produced in appropriate host cells transformed with a vector containing DNA thought to encode a cyclin.

Based on work described herein, it is possible to detect altered expression of a D-type cyclin or increased rates of cell division in cells obtained from a tissue or biological sample, such as blood, urine, feces, mucous or saliva. This has potential for use for diagnostic and prognostic purposes since, for example, there appears to be a link between alteration of a cyclin gene expression and cellular transformation or abnormal cell proliferation. For example, several previous reports have suggested the oncogenic potential of altered human cyclin A function. The human cyclin A gene was found to be a target for hepatitis B virus integration in a hepatocellular carcinoma (Wand, J. et al., Nature 343:555-557 (1990)). Cyclin A has also been shown to associate with adenovirus E1A in virally infected cells (Giordano, A et al., Cell 58:981-990 (1989); Pines, J. and T. Hunter, Nature 346:760-763 (1990)). Further, the PRAD1 gene, which has the same sequence as the cyclin D1 gene, may play an important role in the development of various tumors (e.g., non-parathyroid neoplasis, human breast carcinomas and squamous cell carcinomas) with abnormalities in chromosome 11q13. In particular, identification of CCND1 (PRAD1) as a candidate BCL1 oncogene provides the most direct evidence for the oncogenic potential of cyclin genes. This also suggests that other members of the D-type cyclin family may be involved in oncogenesis. In this context, the chromosomal locations of the CCND2 and CCND3 genes have been mapped to 12p13 and 6p21, respectively. Region 12p13 contains sites of several translocations that are associated with specific immunophenotypes of disease, such as acute lymphoblastic

leukemia, chronic myelomoncytic leukemia, and acute myeloid leukemia. Particularly, the isochromosome of the short arm of chromosome 12 [1(12p)] is one of a few known consistent chromosomal abnormalities in human solid tumors and is seen in 90% of adult testicular germ cell tumors. Region 6p21, on the other hand, has been implicated in the manifestation of chronic lymphoproliferative disorder and leiomyoma. Region tp21, the locus of HLA complex, is also one of the best characterized regions of the human genome. Many diseases have been previously linked to the KLA complex, but the etiology of few of these diseases is fully understood. Molecular cloning and chromosomal localization of cyclins D2 and D3 should make it possible to determine whether they are directly involved in these translocations, and if so, whether they are activated. If they prove to be involved, diagnostic and therapeutic methods described herein can be used to assess an individual's disease state or probability of developing a condition associated with or caused by such translocations, to monitor therapy effectiveness (by assessing the effect of a drug or drugs on cell proliferation) and to provide treatment.

The present invention includes a diagnostic method to detect altered expression of a cyclin gene, such as cyclin D1, D2, D3 or another D-type cyclin. The method can be carried out to detect altered expression in cells or in a biological sample. As shown herein, there is high sequence similarity among cyclin D genes, which indicates that different members of D-type cyclins may use similar mechanisms in regulating the cell cycle (e.g., association with the same catalytic subunit and

acting upon the same substrates). The fact that there is cell-type-specific differential expression, in both mouse and human cells, makes it reasonable to suggest that different cell lineages or different tissues may use different D-type cyclins to perform very similar functions and that altered tissue-specific expression of cyclin D genes as a result of translocation or other mutational events may contribute to abnormal cell proliferation. As described herein, cyclin D1 is expressed differentially in tissues analyzed; in particular, it has been shown to be expressed at the highest levels in cells of neural origin (e.g., glioblastoma cells).

As a result of the work described herein, D-type cyclin expression can be detected and/or quantitated and results used as an indicator of normal or abnormal (e.g., abnormally high rate of) cell division. Differential expression (either expression in various cell types or of one or more of the types of D cyclins) can also be determined.

In a diagnostic method of the present invention, cells obtained from an individual are processed in order to render nucleic acid sequences in them available for hybridization with complementary nucleic acid sequences. All or a portion of the D1, D2 and/or D3 cyclin (or other D-type cyclin gene) sequences can be used as a probe(s). Such probes can be a portion of a D-type cyclin gene; such a portion must be of sufficient length to hybridize to complementary sequences in a sample and remain hybridized under the conditions used and will generally be at least six nucleotides long. Hybridization is detected using known techniques (e.g., measurement of labeled

hybridization complexes, if radiolabeled or fluorescently labeled oligonucleotide probed are used). The extent to which hybridization occurs is quantitated; increased levels of the D-type cyclin gene is indicative of increased potential for cell division.

Alternatively, the extent to which a D-type cyclin (or cyclins) is present in cells, in a specific cell type or in a body fluid can be determined using known techniques and an antibody specific for the D-type cyclin(s). In a third type of diagnostic method, complex formation between the D-type cyclin and the protein kinase with which it normally or typically complexes is assessed, using exogenous substrate, such as histone H1, as a substrate. Arion, D. et al., Cell, 55:371-378 (1988). In each diagnostic method, comparison of results obtained from cells or a body fluid being analyzed with results obtained from an appropriate control (e.g., cells of the same type known to have normal D-type cyclin levels and/or activity or the same body fluid obtained from an individual known to have normal D-type cyclin levels and/or activity) is carried out. Increased D-type cyclin levels and/or activity may be indicative of an increased probability of abnormal cell proliferation or oncogenesis or of the actual occurrence of abnormal proliferation or oncogenesis. It is also possible to detect more than one type of cyclin (e.g., A, B, and/or D) in a cell or tissue sample by using a set of probes (e.g., a set of nucleic acid probes or a set of antibodies), the members of which each recognize and bind to a selected cyclin and collectively provide information about two or more cyclins in the tissues or cells analyzed. Such probes are also

the subject of the present invention; they will generally be detectably labelled (e.g., with a radioactive label, a fluorescent material, biotin or another member of a binding pair or an enzyme).

A method of inhibiting cell division, particularly cell division which would otherwise occur at an abnormally high rate, is also possible. For example, increased cell division is reduced or prevented by introducing into cells a drug or other agent which can block, directly or indirectly, formation of the protein kinase-D type cyclin complex and, thus, block activation of the enzyme. In one embodiment, complex formation is prevented in an indirect manner, such as by preventing transcription and/or translation of the D-type cyclin DNA and/or RNA. This can be carried out by introducing antisense oligonucleotides into cells, in which they hybridize to the cyclin-encoding nucleic acid sequences, preventing their further processing. It is also possible to inhibit expression of the cyclin by interfering with an essential D-type transcription factor. There are reasons to believe that the regulation of cyclin gene transcription may play an important role in regulating the cell cycle and cell growth and oscillations of cyclin mRNA levels are critical in controlling cell division. The G1 phase is the time at which cells commit to a new round of division in response to external and internal sequences and, thus, transcription factors which regulate expression of G1 cyclins are surely important in controlling cell proliferation. Modulation of the transcription factors is one route by which D-type cyclin activity can be influenced, resulting, in the case of inhibition or

prevention of function of the transcription factor(s), in reduced D-type cyclin activity. Alternatively, complex formation can be prevented indirectly by degrading the D-type cyclin(s), such as by introducing a protease or substance which enhances cyclin breakdown into cells. In either case, the effect is indirect in that less D-type cyclin is available than would otherwise be the case.

In another embodiment, protein kinase-D type cyclin complex formation is prevented in a more direct manner by, for example, introducing into cells a drug or other agent which binds the protein kinase or the D-type cyclin or otherwise interferes with the physical association between the cyclin and the protein kinase it activates (e.g., by intercalation) or disrupts the catalytic activity of the enzyme. This can be effected by means of antibodies which bind the kinase or the cyclin or a peptide or low molecular weight organic compound which, like the endogenous D-type cyclin, binds the protein kinase, but whose binding does not result in activation of the enzyme or results in its being disabled or degraded. Peptides and small organic compounds to be used for this purpose can be designed, based on analysis of the amino acid sequences of D-type cyclins, to include residues necessary for binding and to exclude residues whose presence results in activation. This can be done, for example, by systematically mapping the binding site(s) and designing molecules which recognize or otherwise associate with the site(s) necessary for activation, but do not cause activation. As described herein, there is differential expression in tissues of

D-type cyclins. Thus, it is possible to selectively decrease mitotic capability of cells by the use of an agent (e.g., an antibody or anti-sense or other nucleic acid molecule) which is designed to interfere with (inhibit) the activity and/or level of expression of a selected type (or types) of D cyclin. For example, in treating tumors involving the central nervous system or other non-hemotopoietic tissues, agents which selectively inhibit cyclin D1 might be expected to be particularly useful, since D1 has been shown to be differentially expressed (expressed at particularly high levels in cells of neural origin).

Antibodies specifically reactive with D-type cyclins of the present invention can also be produced, using known methods. For example, anti-D type cyclin antisera can be produced by injecting an appropriate host (e.g., rabbits, mice, rats, pigs) with the D-type cyclin against which anti sera is desired and withdrawing blood from the host animal after sufficient time for antibodies to have been formed. Monoclonal antibodies can also be produced using known techniques. Sambrook, J. et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1989).

The present invention also includes a method of screening compounds or molecules for their ability to inhibit or suppress the function of a cyclin, particularly a D-type cyclin. For example, mutant cells as described herein, in which a D-type cyclin such as D1 or D3, is expressed, can be used. A compound or molecule to be assessed for its ability to inhibit a D-type cyclin is contacted with the cells, under conditions appropriate

for entry of the compound or molecule into the cells. Inhibition of the cyclin will result in arrest of the cells or a reduced rate of cell division. Comparison of the rate or extent of cell division in the presence of the compound or molecule being assessed with cell division of an appropriate control (e.g., the same type of cells without added test drug) will demonstrate the ability or inability of the compound or molecule to inhibit the cyclin. Existing compounds or molecules (e.g., those present in a fermentation broth or a chemical "library") or those developed to inhibit the cyclin activation of its protein kinase can be screened for their effectiveness using this method. Drugs which inhibit D-type cyclin are also the subject of this invention.

The present invention will now be illustrated by the following examples, which are not intended to be limiting in any way.

EXAMPLES

Experimental procedures for Examples 1-3 are presented after Example 3.

EXAMPLE 1 Identification of Human cDNA Clones that Rescue CLN Deficiency

In S. cerevisiae, there are three Cln proteins. Disruption of any one CLN gene has little effect on growth, but if all three CLN genes are disrupted, the cells arrest in G1 (Richardson, H.E. et al., Cell 59:1127-1133 (1989)). A yeast strain was constructed, as

described below, which contained insertional mutations in the CLN1 and CLN2 genes to render them inactive. The remaining CLN3 gene was further altered to allow for conditional expression from the galactose-inducible, glucose-repressible promoter GAL1 (see Figure 1). The strain is designated 305-15d #21. In medium containing galactose the CLN3 gene is expressed and despite the absence of both CLN1 and CLN2, cell viability is retained (Fig. 1). In a medium containing glucose, all CLN function is lost and the cells arrest in the G1 phase of the cell cycle.

A human glioblastoma cDNA library carried in the yeast expression vector pADNS (Colicelli, J. et al., Pro. Natl. Acad. Sci. USA 86:3599-3603 (1989)) was introduced into the yeast. The vector pADNS has the LEU2 marker, the 2μ replication origin, and the promoter and terminator sequences from the yeast alcohol dehydrogenase gene (Figure 1). Approximately 3×10^6 transformants were screened for the ability to grow on glucose containing medium. After 12 days of incubation, twelve colonies were obtained. The majority of these proved to be revertants. However, in two cases, the ability to grow on glucose correlated with the maintenance of the LEU2 marker as assessed by plasmid stability tests. These two yeast transformants carried plasmids designated pCYCD1-21 and pCYCD1-19 (see below). Both were recovered in E. coli. Upon reintroduction into yeast, the plasmids rescued the CLN deficient strain, although the rescue was inefficient and the rescued strain grew relatively poorly.

The restriction map and partial DNA sequence analysis revealed that pCYCD1-19 and pCYCD1-21 were independent clones representing the same gene. The 1.2 kb insert of pCYCD1-21 was used as probe to screen a human HeLa cDNA library for a full length cDNA clone. Approximately 2 million cDNA clones were screened and 9 positives were obtained. The longest one of these clones, pCYCD1-H12 (1325 bp), was completely sequenced (Figure 2). The sequence exhibits a very high GC content within the coding region (61%) and contains a poly A tail (69 A residues). The estimated molecular weight of the predicted protein product of the gene is 33,670 daltons starting from the first in-frame AUG codon at nucleotide 145 (Figure 2). The predicted protein is related to other cyclins (see below) and has an unusually low pI of 4.9 (compared to 6.4 of human cyclin A, 7.7 of human cyclin B and 5.6 of CLN1), largely contributed by the high concentration of acidic residues at its C-terminus.

There are neither methionine nor stop codons 5' to the predicted initiating methionine at nucleotide 145. Because of this and also because of the apparent N-terminal truncation of CYCD1 with respect to other cyclins (see below for more detail), four additional human cDNA libraries were further screened to see if the λCYCD1-H12 clone might lack the full 5' region of the cDNA. Among more than 100 cDNA clones isolated from these screens, none was found that had a more extensive 5' region than that of λCYCD1-H12. The full length coding capacity of clone H12 was later confirmed by Western blot analysis (see below).

CYCD1 encodes the smallest (34 kd) cyclin protein identified so far, compared to the 49 kd human cyclin A, 50 kd human cyclin B and 62 kd S. cerevisiae CLN1. By comparison with A and B type cyclins, the difference is due to the lack of almost the entire N-terminal segment that contains the so called "destruction box" identified in both A and B type cyclins (Glotzer, M. et al., Nature 349:132-138 (1991)).

Sequence Analysis of D1 and Comparison with Other Cyclins

Sequence analysis revealed homology between the CYCD1-H12 encoded protein and other cyclins. However, it is clear that CYCD1 differs from the three existing classes of cyclins, A, B and CLN. To examine how this new cyclin gene might be evolutionary related to other cyclins, a comprehensive amino acid sequence comparison of all cyclin genes was conducted. Fifteen previously published cyclin sequences as well as CYCD1 were first aligned using a strategy described in detail by Xiong and Eickbush (Xiong, Y. and T.H. Eickbush, EMBO J. 9:3353-3362 (1990)). Effort was made to reach the maximum similarity between sequences with the minimum introduction of insertion/deletions and to include as much sequence as possible. With the exception of CLN cyclins, this alignment contains about 200 amino acids residues which occupies more than 70% of total coding region of CYCD1 (Figure 5A). There is a conserved domain and some scattered similarities between members of A and B type cyclins N-terminal to the aligned region (Glotzer, M. et al., Nature 349:132-138 (1991)), but this is not

present in either CLN cyclins or CYCD1 and CYL1 and so they were not included in the alignment.

The percent divergence for all pairwise comparisons of the 17 aligned sequences was calculated and used to construct an evolutionary tree of cyclin gene family using the Neighbor-Joining method (Saitou, N. and M. Nei, Mol. Biol. Evol. 4:406-425 (1987) and Experimental Procedures). Because of the lowest similarity of CLN cyclins to the other three classes, the tree (Figure 5B) was rooted at the connection between the CLN cyclins and the others. It is very clear from this evolutionary tree that CYCD1, CYCD2 and CYCD3 represent a distinct new class of cyclin, designated cyclin D.

EXAMPLE 2 Expression of the Cyclin D1 Gene in Human Cells

Expression of cyclin D1 gene in human cells was studied by Northern analysis. Initial studies indicated that the level of cyclin D1 expression was very low in several cell lines. Poly (A)+RNA was prepared from HeLa cells and probed with the entire coding region of CYCD1 gene. Two major transcripts of 4.8 kb and 1.7 kb were detected. The high molecular weight form was the most abundant. With the exception of a few cDNA clones, which were truncated at either the 5′ or 3′ ends, most of the cDNA clones isolated from various different cDNA libraries are very similar to the clone λCYCD1-H12 (Figure 2). Thus, it appears that the 1.7 kb transcript detected in Northern blots corresponds to nucleotide sequence in Figure 2.

To understand the origin of the larger 4.8 kb transcript, both 5' and 3' end sub-fragments of the λCYCD1-H12 clone were used to screen both cDNA and genomic libraries, to test whether there might be alternative transcription initiation, polyadenylation and/or mRNA splicing. Two longer cDNA clones, λCYCD1-H034 (1.7 kb) from HeLa cells and λDYDC1-T078 (4.1 kb) from human teratocarcinoma cells, as well as several genomic clones were isolated and partially sequenced. Both λCYCD1-H034 and λCYCD1-T078 have identical sequences to λCYCD1-H12 clone from their 5' ends (Figure 6). Both differ from λCYCD1-H12 in having additional sequences at the 3' end, after the site of polyadenylation. These 3' sequences are the same in λCYCD1-H034 and λCYCD1-T078, but extend further in the latter clone (Figure 6). Nucleotide sequencing of a genomic clone within this region revealed colinearity between the cDNAs and the genomic DNA (Figure 6). There is a single base deletion (an A residue) in λCYCD1-T078 cDNA clone. This may be the result of polymorphism, although it is not possible to exclude the possibility that some other mechanism is involved. The same 4.8 kb transcript, but not the 1.7 kb transcript, was detected using the 3' end extra fragment from clone T078 as a probe.

It appears that the two mRNAs detected in Northern blots arise by differential polyadenylation (Figure 6). Strangely, there is no recognizable polyadenylation sequence (AAUAAA) anywhere within the sequence of clone λCYCD1-H12, even though polyadenylation has clearly occurred (Figure 2). There is also no close variant of AAUAAA (nothing with less than two mismatches).

EXAMPLE 3 Differential Expression of Cyclin D1 Gene in Different Cell Types

During the screening of cDNA libraries to obtain full length clones of CYCD1, it became evident that the cDNA library derived from the human glioblastoma cell line (U118 MG) from which the yeast transformants were obtained gave rise to many more positives than the other four cDNA libraries. Northern and Western blotting were carried out to explore the possibility that cyclin D1 might be differentially expressed in different tissues or cell lines. Total RNA was isolated from U118 MG cells and analyzed by Northern blot using the CYCD1 gene coding region as probe. The level of transcript is 7 to 10 fold higher in the glioblastoma cells, compared to HeLa cells. In both HeLa and U118 MG cells, both high and low molecular weight transcripts are observed.

To investigate whether the abundant CYCD1 message in the U118 MG cell line is reflected at the protein level, cell extracts were prepared and Western blotting was performed using anti-CYL1 prepared against mouse CYL1 (provided by Matsushime, H. et al.). This anti-CYL1 antibody was able to detect nanogram quantities of recombinant CYCD1 on Western blots (data not shown), and was also able to detect CYCD1 in the original yeast transformants by immunoprecipitation and Western analysis. Initial experiments using total cell extracts, from HeLa, 293 or U118 MG cells failed to detect any signal. However, if the cell extracts were immuno-precipitated with the serum before being subjected to SDS-PAGE and immunoblotting, a 34 kd polypeptide was readily detected in U118 MG cells. The protein is far

less abundant in HeLa cells and was not detectable in 293 cells. The molecular weight of the anti-CYCL1 cross-reactive material from U118 MG and HeLa is exactly that of the human CYCD1 protein expressed in E. coli. This argues that the sequenced cDNA clones contain the entire open reading frame.

EXPERIMENTAL PROCEDURES

Strain Construction

The parental strain was BF305-15d (MATa leu2-3 leu2-112 his3-11 his3-15 ura3-52 trp1 ade1 met14 arg5,6) (Futcher, B. and J. Carbon, Mol. Cell. Biol. 6:2213-2222 (1986)). The strain was converted into a conditional cln- strain in three steps. First, the chromosomal CLN3 gene was placed under control of the GAL1 promoter. A 0.75 kb EcoRI-BamHI fragment containing the bidirectional GAL10-GAL1 promoters was fused to the 5' end of the CLN3 gene, such that the BamHI (GAL1) end was attached 110 nucleotides upstream of the CLN3 start codon. An EcoRI fragment stretching from the GAL10 promoter to the middle of CLN3 (Nash, R. et al., EMBO J. 7:4335-4346 (1988)) was then subcloned between the XhoI and EcoRI sites of pBF30 (Nash, R. et al., EMBO J. 7:4335-4346 (1988)). The ligation of the XhoI end to the EcoRI end was accomplished by filling in the ends with Klenow, and blunt-end ligating (destroying the EcoRI site). As a result, the GAL1 promoter had replaced the DNA normally found between -110 and -411 upstream of CLN3. Next, an EcoRI to SphI fragment was excised from this new pBF30 derivative. This fragment had extensive 5' and 3' homology to the

CLN3 region, but contained the GAL1 promoter and a URA3 marker just upstream of CLN3. Strain BF305-15d was transformed with this fragment and Ura+ transformants were selected. These were checked by Southern analysis. In addition, average cell size was measured when the GAL1 promoter was induced or uninduced. When the GAL1 promoter was induced by growing the cells in 1% raffinose and 1% galactose, mode cell volume was about 25μm^3 (compared to a mode volume of about 40μm^3 for the parental strain) whereas when the promoter was not induced (raffinose alone), or was repressed by the presence of glucose, cell volume was much larger than for the wild-type strain. These experiments showed that CLN3 had been placed under control of the GAL1 promoter. It is important to note that this GAL1-controlled, glucose repressible gene is the only source of CLN3 protein in the cell.

Second, the CLN1 gene was disrupted. A fragment of CLN1 was obtained from I. Fitch, and used to obtain a full length clone of CLN1 by hybridization, and this was subcloned into a pUC plasmid. A BamHI fragment carrying the HIS3 gene was inserted into an NcoI site in the CLN1 open reading frame. A large EcoRI fragment with extensive 5' and 3' homology to the CLN1 region was then excised, and used to transform the BF305-15d GAL-CLN3 strain described above. Transformation was done on YNB-his raffinose galactose plates. His+ clones were selected, and checked by Southern analysis.

Finally, the CLN2 gene was disrupted. A fragment of CLN2 was obtained from I. Fitch, and used to obtain a full length clone of CLN2 by hybridization, and this was

subcloned into a pUC plasmid. An EcoRI fragment carrying the TRP1 gene was inserted into an SpeI site in the CLN2 open reading frame. A BamHI-KpnI fragment was excised and used to transform the BF305-15d GAL-CLN3 HIS3::cln1 strain described above. Transformation was done on YNB-trp raffinose galactose plates. Trp+ clones were selected. In this case, because the TRP1 fragment included an ARS, many of the transformants contained autonomously replicating plasmid rather than a disrupted CLN2 gene. However, several percent of the transformants were simple TRP1::cln2 disruptants, as shown by phenotypic and Southern analysis.

One particular 305-15d GAL1-CLN3 HIS3::cln1 TRP1::cln2 transformant called clone #21 (referred to hereafter as 305-15d #21) was analyzed extensively. When grown in 1% raffinose and 1% galactose, it had a doubling time indistinguishable from the CLN wild-type parental strain. However, it displayed a moderate Wee phenotype (small cell volume), as expected for a CLN3 overexpressor. When glucose was added, or when galactose was removed, cells accumulated in G1 phase, and cell division ceased, though cells continued to increase in mass and volume. After overnight incubation in the G1-arrested state, essentially no budded cells were seen, and a large proportion of the cells had lysed due to their uncontrolled increase in size.

When 305-15d #21 was spread on glucose plates, revertant colonies arose at a frequency of about 10-7. The nature of these glucose-resistant, galactose-independent mutants was not investigated.

Yeast Spheroplasts Transformation

S. cerevisae spheroplasts transformation was carried out according to Burgers and Percival and Allshire (Burgers, P.M.J. and K.J. Percival, Anal. Biochem. 163:391-397 (1987); Allshire, R.C., Proc. Natl. Acad. Sci. USA 87:4043-4047 (1990)).

Cell Culture

HeLa and 293 cells were cultured at 37°C either on plates or in suspension in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum. Glioblastoma U118 MG cells were cultured on plates in DMEM supplemented with 15% fetal bovine serum and 0.1 mM non-essential amino acid (GIBCO).

Nucleic Acid Procedures

Most molecular biology techniques were essentially the same as described by Sambrook et al. (Sambrook, J. et al., Molecular Cloning: A Laboratory Manual Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1989)). Phagmid vectors pUC118 or pUC119 (Vieira, J. and J. Messing, Meth. Enzymol. 153:3-11 (1987)) or pBlueScript (Stratagene) were used as cloning vectors. DNA sequences were determined either by a chain termination method (Sanger, F. et al., Proc. Natl. Acad. Sci. USA 74:5463-5467 (1977)) using Sequenase Kit (United States Biochemical) or on an Automated Sequencing System (373A, Applied Biosystems).

Human HeLa cell cDNA library in λZAP II was purchased from Stratagene. Human T cell cDNA library in λgt10 was a gift of M. Gillman (Cold Spring Harbor

Laboratory). Human glioblastoma U118 MG and glioblastoma SW1088 cell cDNA libraries in λZAP II were gifts of M. Wigler (Cold Spring Harbor Laboratory). Human teratocarcinoma cell cDNA library λgt10 was a gift of Skowronski (Cold Spring Harbor Laboratory). Normal human liver genomic library λGEM-11 was purchased from Promega.

Total RNA from cell culture was extracted exactly according to Sambrook et al. (Sambrook, J. et al., Molecular Cloning: A Laboratory Manual Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1989)) using guanidium thiocyanate followed by centrifugation in CsCl solution. Poly(A)+RNA was isolated from total RNA preparation using Poly (A)+Quick push columns (Stratagene). RNA samples were separated on a 1% agarose-formaldehyde-MOPS gel and transferred to a nitrocellulose filter. Northern hybridizations (as well as library screening) were carried out at 68°C in a solution containing 5 x Denhardt's solution, 2 x SSC, 0.1% SDS, 100 μg/ml denatured Salmon sperm DNA, 25 μM $NaPO_4$ (pH7.0) and 10% dextran sulfate. Probes were labelled by the random priming labelling method (Feinberg, A. and B. Vogelstein, Anal. Biochem. 132:6-13 (1983)). A 1.3 kb Hind III fragment of cDNA clone pCYCD1-H12 was used as coding region probe for Northern hybridization and genomic library screening, a 1.7 kb Hind III-EcoRI fragment from cDNA clone pCYCD1-T078 was used as 3' fragment probe.

To express human cyclin D1 gene in bacteria, a 1.3 kb Nco I-Hind II fragment of pCYCD1-H12 containing the entire CYCD1 open reading frame was subcloned into a T7 expression vector (pET3d, Studier, F.W. et al., Methods in Enzymology 185:60-89 (1990)). Induction of E. coli

strain BL21 (DE3) harboring the expression construct was according to Studier (Studier, F.W. et al., Methods in Enzymology 185:60-89 (1990)). Bacterial culture was lysed by sonication in a lysis buffer (5 mM EDTA, 10% glycerol, 50 mM Tris-HCL, pH 8.0, 0.005% Triton X-100) containing 6 M urea (CYCD1 encoded p34 is only partial soluble in 8 M urea), centrifuged for 15 minutes at 20,000 g force. The pellet was washed once in the lysis buffer with 6 M urea, pelleted again, resuspended in lysis buffer containing 8 M urea, and centrifuged. The supernatant which enriched the 34 kd CYCD1 protein was loaded on a 10% polyacrymide gel. The 34 kd band was cut from the gel and eluted with PBS containing 0.1% SDS.

Sequence Alignment and Formation of an Evolutionary Tree

Protein sequence alignment was conducted virtually by eye according to the methods described and discussed in detail by Xiong and Eickbush (Xiong, Y. and T.H. Eickbush, EMBO J. 9:3353-3362 (1990)). Numbers within certain sequences indicate the number of amino acid residues omitted from the sequence as the result of insertion.

Numbers within certain sequences indicate the number of amino acid residues omitted from the sequence as the result of insertion (e.g., for CLN1, ...TWG25RLS....- indicates that 25 amino acids have been omitted between G and R). Sources for each sequence used in this alignment and in the construction of an evolutionary tree (Figure 5B) are as follows: CYCA-Hs, human A type cyclin (Wang, J. et al., Nature 343:555-557 (1990)); CYCA-X1, Xenopus A-type cyclin (Minshull, J. et al., EMBO J. 9:2865-2875

(1990)); CYCA-Ss, clam A-type cyclin (Swenson, K.I. et al., Cell 47:867-870 (1986); CYCA-Dm, Drosophila A-type cyclin (Lehner, C.F. and P.H. O'Farrell, Cell 56:957-968 (1989)); CYCB1-Hs, human B1-type cyclin (Pines, J. and T. Hunter, Cell 58:833-846 (1989)); CYCB1-X1 and CYCB2-X1, Xenopus B1- and B2-type cyclin (Minshull, J. et al., Cell 56:947-956 (1989)); CYCB-Ss, clam B-type cyclin (Westendorf, J.M et al., J. Cell Biol., 108:1431-1444 (1989)); CYCB-Asp, starfish B-type cyclin (Tachibana, K. et al., Dev. Biol. 140:241-252 (1990)); CYCB-Arp, sea urchin B-type cyclin (Pines, J. and T. Hunter, EMBO J. 6:2987-2995 (1987)); CYCB-Dm, Drosophila B-type cyclin (Lehner, C.F. and P.H. O'Farrell, Cell 61:535-547 (1990)); CDC13-Sp, S. pombe CDC13 (Booher, R. and D. Beach, EMBO J. 7:2321-2327 (1988)); CLN1-Sc and CLN2-Sc, S. cerevisiae cyclin 1 and 2 (Hadwiger, J.A. et al., Proc. Natl. Acad. Sci. USA 86:6255-6259 (1989)); CLN3-Sc, S. cerevisiae cyclin 3 (Nash, R. et al., EMBO J. 7:4335-4346 (1988)).

A total of 17 cyclin sequences were aligned and two representive sequences from each class are presented in Figure 5A.

Percent divergence of all pairwise comparison of 17 sequences were calculated from 154 amino acid residues common to all 17 sequences, which does not include the 50 residue segments located at N-terminal part of A, B and D-type cyclins because of its absence from CLN type cyclins. A gap/insertion was counted as one mismatch regardless of its size. Before tree construction, all values were changed to distance with Poisson correction ($d = -\log_e S$, where the S = sequence similarity (Nei, M.,

Molecular Evolutionary Genetics pp. 287-326 Columbia University Press, NY (1987)). Calculation of pairwise comparison and Poisson correction were conducted using computer programs developed at University of Rochester. Evolutionary trees of cyclin gene family was generated by the Neighbor-Joining program (Saitou, N. and M. Nei, Mol. Biol. Evol. 4:406-567 (1987)). All calculations were conducted on VAX computer MicroVMS V4.4 of Cold Spring Harbor Laboratory. The reliability of the tree was evaluated by using a subset sequence (e.g., A, B and D-type cyclins), including more residues (e.g., the 50-residue segment located at C-terminal of A, B and D-type cyclins, Figure 5A) or adding several other unpublished cyclin sequences. They all gave rise to the tree with the same topology as the one presented in Figure 5B.

Immunoprecipitation and Western Blots

Cells from 60 to 80% confluent 100 mm dish were lysed in 1 ml of lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 20 mM EDTA, 0.5% NP-40, 0.5% Nadeoxycholate, 1 mM PMSF) for 30 minutes on ice. Immunoprecipitation was carried out using 1 mg protein from each cell lysate at 4°C for overnight. After equilibrated with the lysis buffer, 60 μl of Protein A-agarose (PIERCE) was added to each immunoprecipitation and incubated at 4°C for 1 hour with constant rotating. The immunoprecipitate was washed three times with the lysis buffer and final resuspended in 50 μl 2 x SDS protein sample buffer, boiled for 5 minutes and loaded onto a 10% polyacrymide gel. Proteins were transferred to a nitrocellulose filter using a SDE Electroblotting System (Millipore) for 45 minutes at a

constant current of 400 mA. The filter was blocked for 2 to 6 hours with 1 x PBS, 3% BSA and 0.1% sodium azide, washed 10 minutes each time and 6 times with NET gel buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% NP-40, 1 mM EDTA, 0.25% gelatin and 0.02 sodium azide), radiolabelled with ^{125}I-Protein A for 1 hour in blocking solution with shaking. The blot was then washed 10 minutes each time and 6 times with the NET gel buffer before autoradiography.

The tree was constructed using the Neighbor-Joining method (Saitou, N. and M. Nei, Mol. Biol. Evol., 4:406-425 (1987). The length of horizontal line reflects the divergence. The branch length between the node connecting the CLN cyclins and other cyclins was arbitrary divided.

MATERIALS AND METHODS

The following materials and methods were used in the work described in Examples 4-6.

Molecular Cloning

The human HeLa cell cDNA library, the human glioblastoma cell U118 MG cDNA library, the normal human liver genomic library, and the hybridization buffer were the same as those described above. A human hippocampus cDNA library was purchased from Stratagene, Inc. High- and low-stringency hybridizations were carried out at 68° and 50°C, respectively. To prepare template DNA for PCR reactions, approximately 2 million lambda phages from each cDNA library were plated at a density of 10^5 PFU/150-mm plate, and DNA was prepared from the plate lysate

according to Sambrook, J. et al., Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1989.

EXAMPLE 4 Isolation of Human Cyclin D2 and D3 cDNAs

To isolate human cyclin D2 and D3 cDNAs, two 5' oligonucleotides and one 3' degenerate oligonucleotide were derived from three highly conserved regions of human CCND1, mouse cyl1, cyl2, and cyl3 D-type cyclins (Matsushime, H. et al., Cell 65:701-713 (1991); Xiong, Y. et al., Cell 65:691-699; Fig. 8). The first 5' oligonucleotide primer, HCND11, is a 8192-fold degenerate 38-mer (TGGATG[T/C]TNGA[A/G]GTNTG[T/C]GA[A/C]GA[A/G]CA-[A/G]AA[A/G]TG[T/C]GA[A/G]GA)(SEQ ID No. 37), encoding 13 amino acids (WMLEVCEEQKCEE)(SEQ ID No. 38). The second 5' oligonucleotide primer, HCND12, is a 8192-fold degenerate 29-mer (GTNTT[T/C]CCN[T/C]TNGCNATGAA[T/C]TA[T/C]-TNGA)(SEQ ID No. 39), encoding 10 amino acids (VFPLAMNYLD)(SEQ ID No. 40). The 3' primer, HCND13, is a 3072-fold degenerate 24-mer ([A/G]TCNGT[A/G]TA[A/G/T]AT-[A/G]CANA[A/G][T/C]TT-[T/C]TC)(SEQ ID No. 41), encoding 8 amino acids (EKLCIYTD)(SEQ ID No. 42). The PCR reactions were carried out for 30 cycles at 94°C for 1 min, 48°C for 1 min, and 72°C for 1 min. The reactions contained 50 mM KCl, 10 mM Tris-HCl(pH 8.3), 1.5 mM $MgCl_2$, 0.01% gelatin, 0.2 mM each of dATP, dGTP, dCTP, and dTTP, 2.5 units of Taq polymerase, 5 μM of oligonucleotide, and 2-10 μg of template DNA. PCR products generated by HCND11 and HCND13 were verified in a second-round PCT reaction using HCND12 and HCND13 as the primers. After resolution on a 1.2% agarose gel, DNA fragments with the

expected size (200 bp between primer HCND11 and HCND13) were purified and subcloned into the SmaI site of phagmid vector pUC118 for sequencing.

To isolate full-length cyclin D3 cDNA, the 201-bp fragment of the D3 PCR product was labeled with oligonucleotide primers HCND11 and HCND13 using a random-primed labeling technique (Feinberg, A. P. et al., Anal. Biochem. 132:6-13 (1983)) and used to screen a human HeLa cell cDNA library. The probe used to screen the human genomic library for the CCND3 gene was a 2-kb EcoRI fragment derived from cDNA clone λD3-H34. All hybridizations for the screen of human cyclin D3 were carried out at high stringency.

The PCR clones corresponding to CCND1 and CCND3 have been repeatedly isolated from both cDNA libraries; CCND2 has not. To isolate cyclin D2, a 1-kb EcoRI fragment derived from mouse cyl2 cDNA was used as a probe to screen a human genomic library. Under low-stringency conditions, this probe hybridized to both human cyclins D1 and D2. The cyclin D1 clones were eliminated through another hybridization with a human cyclin D1 probe at high stringency. Human CCND2 genomic clones were subsequently identified by partial sequencing and by comparing the predicted protein sequence with that of human cyclins D1 and D3 as well as mouse cyl2.

As described above, human CCND1 (cyclin D1) was isolated by rescuing a triple Cln deficiency mutant of Saccharomyces cerevisiae using a genetic complementation screen. Evolutionary proximity between human and mouse, and the high sequence similarity among cyl1, cyl2, and cyl3, suggested the existence of two additional D-type

cyclin genes in the human genome. The PCR technique was first used to isolate the putative human cyclin D2 and D3 genes. Three degenerate oligonucleotide primers were derived from highly conserved regions of human CCND1, mouse cyl1, cyl2, and cyl3. Using these primers, cyclin D1 and a 200-bp DNA fragment that appeared to be the human homolog of mouse cyl3 from both human HeLa cell and glioblastoma cell cDNA libraries was isolated. A human HeLa cell cDNA library was screened with this PCR product as probe to obtain a full-length D3 clone. Some 1.2 million cDNA clones were screened, and six positives were obtained. The longest cDNA clone from this screen, λD3-H34 (1962 bp), was completely sequenced (Figure 4).

Because a putative human cyclin D2 cDNA was not detected by PCR, mouse cyl2 cDNA was used as a heterologous probe to screen a human cDNA library at low stringency. This resulted, initially, in isolation of 10 clones from the HeLa cell cDNA library, but all corresponded to the human cyclin D1 gene on the basis of restriction mapping. Presumably, this was because cyclin D2 in HeLa cells is expressed at very low levels. Thus, the same probe was used to screen a human genomic library, based on the assumption that the representation of D1 and D2 should be approximately equal. Of the 18 positives obtained, 10 corresponded to human cyclin D1 and 8 appeared to contain human cyclin D2 sequences (see below). A 0.4-kb BamHI restriction fragment derived from λD2-G1 1 of the 8 putative cyclin D2 clones, was then used as probe to screen a human hippocampus cDNA library at high stringency to search for a full-length cDNA clone of the cyclin D2 gene. Nine positives were obtained

after screening of approximately 1 million cDNA clones. The longest cDNA clone, λD2-P3 (1911 bp), was completely sequenced (Figure 3). Neither λD2-P3 nor λD3-H34 contains a poly(A) sequence, suggesting that part of the 3' untranslated region might be missing.

The DNA sequence of λD2-P3 revealed an open reading frame that could encode a 289-amino-acid protein with a 33,045-Da calculated molecular weight. A similar analysis of λD3-H34 revealed a 292-amino-acid open reading frame encoding a protein with a 32,482-Da calculated molecular weight. As in the case of human cyclin D1, there is neither methionine nor stop codons 5' to the presumptive initiating methionine codon for both λD2-P3 (nucleotide position 22, Figure 3) and λD3-H34 (nucleotide position 101, Figure 4). On the basis of the protein sequence comparison with human cyclin D1 and mouse cyl1 (Figure 7) and preliminary results of the RNase protection experiment, both λD2-P3 and λD3-H34 are believed to contain full-length coding regions.

The protein sequence of all 11 mammalian cyclins identified to date were compared to assess their structural and evolutionary relationships. This includes cyclin A, cyclins B1 and B2, six D-type cyclins (three from human and three from mouse), and the recently identified cyclins E and C (Figure 7). Several features concerning D-type cyclins can be seen from this comparison. First, as noted previously for cyclin D1, all three cyclin D genes encode a similar small size protein ranging from 289 to 295 amino acid residues, the shortest cyclins found so far. Second, they all lack the so-called "destruction box" identified in the N-terminus of

both A- and B-type cyclins, which targets it for ubiquitin-dependent degradation (Glotzer, M. et al., Nature 349:132-138 (1991)). This suggests either that the D-type cyclins have evolved a different mechanism to govern their periodic degradation during each cell cycle or that they do not undergo such destruction. Third, the three human cyclin D genes share very high similarity over their entire coding region:60% between D1 and D2, 60% between D2 and D3, and 52% between D1 and D3. Fourth, members of the D-type cyclins are more closely related to each other than are members of the B-type cyclins, averaging 78% for three cyclin D genes in the cyclin box versus 57% for two cyclin B genes. This suggests that the separation (emergence) of D-type cyclins occurred after that of cyclin B1 from B2. Finally, using the well-characterized mitotic B-type cyclin as an index, the most closely related genes are cyclin A (average 51%), followed by the E-type (40%), D-type (29%), and C-type cyclins (20%).

EXAMPLE 5 Chromosome Localization of CCND2 and CCND3

The chromosome localization of CCND2 and CCND3 was determined by fluorescence in situ hybridization. Chromosome in situ suppression hybridization and in situ hybridization banding were performed as described previously (Lichter, T. et al., Science 247:64-69 (1990); Baldini, A. et al., Genomics 9:770-774 (1991)). Briefly λD2-G4 and λD3-G9 lambda genomic DNAs containing inserts of 15 and 16 kb, respectively, were labeled with biotin-11-dUTP (Sigma) by nick-translation (Brigatti, D. J. et

al., Urology 126:32-50 (1983); Boyle, A. L., In Current Protocols in Molecular Biology, Wiley, New York, 1991). Probe size ranged between 200 and 400 nucleotides, and unincorporated nucleotides were separated from probes using Sephadex G-50 spin columns (Sambrook, J. et al., Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1989). Metaphase chromosome spreads prepared by the standard technique (Lichter, T. et al., Science 247:64-69 (1990)) were hybridized in situ with biotin-labeled D2-G4 or D3-G9. Denaturation and preannealing of 5 μg of DNase-treated human placental DNA, 7 μg of DNased salmon sperm DNA, and 100 ng of labeled probe were performed before the cocktail was applied to Alu prehybridized slides. The in situ hybridization banding pattern used for chromosome identification and visual localization of the probe was generated by cohybridizing the spreads with 40 ng of an Alu 48-mer oligonucleotide. This Alu oligo was chemically labeled with digoxigenin-11-dUTP (Boehringer-Mannheim) and denatured before being applied to denatured chromosomes. Following 16-18 h of incubation at 37°C and posthybridization wash, slides were incubated with blocking solution and detection reagent (Lichter, T. et al., Science 247:64-69 (1990)). Biotin-labeled DNA was detected using fluorescence isothiocyanate (FITC)-conjugated avidin DCS (5 μg/ml) (Vector Laboratories); digoxigenin-labeled DNA was detected using a rhodamine-conjugated anti-digoxigenin antibody (Boehringer-Mannheim). Fluorescence signals were imaged separately using a Zeiss Axioskop-20 epifluorescence microscope equipped with a cooled CCD camera (Photometrics CH220).

Camera control and image acquisition were performed using an Apple Macintosh IIX computer. The gray scale images were pseudocolored and merged electronically as described previously (Baldini, A. et al., Genomics 9:770-774 (1991)). Image processing was done on a Macintosh IIci computer using Gene Join Maxpix (software by Tim Rand in the laboratory of D. Ward, Yale) to merge FITC and rhodamine images. Photographs were taken directly from the computer monitor.

Chromosomal fluorescence in situ hybridization was used to localize D2-G4 and D3-G9. The cytogenetic location of D2-G4 on chromosome 12p band 13 and that of D3-G9 on chromosome 6p band 21 were determined by direct visualization of the two-color fluorescence in situ hybridization using the biotin-labeled probe and the digoxigen-labeled Alu 48-mer oligonucleotide (Fig. 5).

The Alu 48-mer R-bands, consistent with the conventional R-banding pattern, were imaged and merged with images generated from the D2-G4 and D3-G9 hybridized probes. The loci of D2-G4 and D3-G9 were visualized against the Alu banding by merging the corresponding FITC and rhodamine images. This merged image allows the direct visualization of D2-G4 and D3-G9 on chromosomes 12 and 6, respectively. The D2-G4 probe lies on the positive R-band 12p13, while D3-G9 lies on the positive R-band 6p21.

Cross-hybridization was not detected with either pseudogene cyclin D2 or D3, presumably because the potentially cross-hybridizing sequence represents only a sufficiently small proportion of the 15- and 16-kb genomic fragments (nonsuppressed) used as probe, and the

nucleotide sequences of pseudogenes have diverged from their ancestral active genes.

EXAMPLE 6 Isolation and Characterization of Genomic Clones of Human D-Type Cyclins

Genomic clones of human D-type cyclins were isolated and characterized to study the genomic structure and to obtain probes for chromosomal mapping. The entire 1.3-kb cyclin D1 cDNA clone was used as probe to screen a normal human liver genomic library. Five million lambda clones were screened, and three positives were obtained. After initial restriction mapping and hybridizations, lambda clone G6 was chosen for further analysis. A 1.7-kb BamHI restriction fragment of λD1-G6 was subcloned into pUC118 and completely sequenced. Comparison with the cDNA clones previously isolated and RNase protection experiment results (Withers, D.A. et al., Mol. Cell. Biol. 11:4846-4853 (1991)) indicated that this fragment corresponds to the 5' part of the cyclin D1 gene. As shown in Figure 8A, it contains 1150 bp of upstream promoter sequence and a 198-bp exon followed by an intron.

Eighteen lambda genomic clones were isolated from a similar screening using mouse cyl2 cDNA as a probe under low-stringency hybridization conditions, as described above (Example 4). Because it was noted in previous cDNA library screening that the mouse cyl2 cDNA probe can cross-hybridize with the human D1 gene at low stringency, a dot-blot hybridization at high stringency was carried out, using the human D1 cDNA probe. Ten of the 18 clones hybridized with the human D1 probe and 8 did not. On the basis of the restriction digestion analysis, the 8 lambda

clones that did not hybridize with the human D1 probe at high stringency fall into three classes respresented by λD2-G1, λD2-G2, and λD2-G4, respectively. These three lambda clones were subcloned into a pUC plasmid vector, and small restriction fragments containing coding region were identified by Southern hybridization using a mouse cyl2 cDNA probe. A 0.4-kb BamHI fragment derived from λD2-G1 was subsequently used as a probe to screen a human hippocampus cell cDNA library at high stringency. Detailed restriction mapping and partial sequencing indicated that λD2-G1 and λD2-G2 were two different clones corresponding to the same gene, whereas λD2-G4 appeared to correspond to a different gene. A 2.7-kb SacI-SmaI fragment from λD2-G4 and 1.5-kb BclI-BglII fragment from λD2-G1 have been completely sequenced. Nucleotide sequence comparison revealed that the clone λD2-G4 corresponds to the D2 cDNA clone λD2-P3 (Figure 3). As shown in Figure 8A, the 2.7-kb SacI-SmaI fragment contains 1620 bp of sequence 5′ to the presumptive initiating methionine codon identified in D2 cDNA (Figure 3) and a 195-bp exon followed by a 907-bp intervening sequence.

Lambda genomic clones corresponding to the human cyclin D3 were isolated from the same genomic library using human D3 cDNA as a probe. Of four million clones screened, nine were positives. Two classes of clones, represented by λD3-G4 and λD3-G9, were distinguished by restriction digestion analysis. A 2.0-kb HindIII-ScaI restriction fragment from λD3-G5 and a 3.7-kb SacI-HindIII restriction fragment from λD3-G9 were further subcloned into a pUC plasmid vector for more detailed

restriction mapping and complete sequencing, as they both hybridized to the 5' cyclin D3 cDNA probe. As presented in Figure 9C, the 3.7-kb fragment from clone G9 contains 1.8 kb of sequence 5' to the presumptive initiating methionine codon identified in D3 cDNA (Figure 4), a 198-bp exon 1, a 684-bp exon 2, and a 870-bp intron.

Comparison of the genomic clones of cyclins D1, D2, and D3 revealed that the coding regions of all three human CCND genes are interrupted at the same position by an intron (indicated by an arrow in Figure 8). This indicated that the intron occurred before the separation of cyclin D genes.

EXAMPLE 7 Isolation and Characterization of Two Cyclin D Pseudogenes

The 1.5-kb BclI-BglII fragment subcloned from clone λD2-G1 has been completely sequenced and compared with cyclin D2 cDNA clone λD2-P3. As shown in Figure 10, it contains three internal stop codons (nucleotide positions 495, 956, and 1310, indicated by asterisks), two frame-shifts (position 1188 and 1291, slash lines), one insertion, and one deletion. It has also accumulated many missense nucleotide substitutions, some of which occurred at the positions that are conserved in all cyclins. For example, triplet CGT at position 277 to 279 of D2 cDNA (Figure 3) encodes amino acid Arg, which is an invariant residue in all cyclins (see Figure 8). A nucleotide change from C to T at the corresponding position (nucleotide 731) in clone D2-G1 (Figure 10) gave rise to a triplet TGT encoding Cys instead of Arg. Sequencing of the 2.0-kb HindIII-ScaI fragment from clone

λD3-G5 revealed a cyclin D3 pseudogene (Figure 11). In addition to a nonsense mutation (nucleotide position 1265), two frameshifts (position 1210 and 1679), a 15-bp internal duplication (underlined region from position 1361 to 1376), and many missense mutations, a nucleotide change from A to G at position 1182 resulted in an amino acid change from the presumptive initiating methionine codon ATG to GTG encoding Val. On the basis of these analyses, we conclude that clones λD2-G1 and λD3-G5 contain pseudogenes of cyclins D2 and D3, respectively.

EQUIVALENTS

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

International Application No: PCT/

MICROORGANISMS

Optional Sheet in connection with the microorganism referred to on page 12, line 28 of the description

A. IDENTIFICATION OF DEPOSIT

Further deposits are identified on an additional sheet ☐

Name of depositary institution

American Type Culture Collection

Address of depositary institution (including postal code and country)

12301 Parklawn Drive
Rockville, Maryland 20852
United States of America

Date of deposit	Accession Number
May 14, 1991	68620

B. ADDITIONAL INDICATIONS (leave blank if not applicable). This information is continued on a separate attached sheet ☐

In respect of those designations in which a European Patent is sought, the Applicant hereby informs the European Patent Office under European Rule 28(4) that, until the publication of the mention of the grant of the European Patent or until the date on which the European Application has been refused or is withdrawn or is deemed to be withdrawn, the availability of the biological material deposited with the American Type Culture Collection under Accession No. 68620 shall be effected only by the issue of a sample to an expert nominated by the requester in accordance with European Rule 28(5).

C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States)

EP (Europe)
CA (Canada)
JP (Japan)

D. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable)

The indications listed below will be submitted to the International Bureau later (Specify the general nature of the indications e.g., "Accession Number of Deposit")

Accession Number of Deposit 68620

E. ☐ This sheet was received with the international application when filed (to be checked by the receiving Office)

Benjamin Levi for
(Authorized Officer)

☐ The date of receipt (from the applicant) by the International Bureau

was

(Authorized Officer)

(January 1985)

Form PCT/R /134 (January 1981)

CLAIMS

1. Recombinant cyclin of mammalian origin which replaces a CLN-type protein essential for cell start in budding yeast.

2. Recombinant cyclin of Claim 1 which is D-type cyclin.

3. Recombinant cyclin of Claim 2 which is of human origin.

4. Recombinant D type cyclin of Claim 3 selected from the group consisting of: cyclin D1, cyclin D2 and cyclin D3.

5. Purified D-type cyclin of mammalian origin of approximate molecular weight 34 kD.

6. Purified D type cyclin of Claim 5 having the amino acid sequence of Figure 2, the amino acid sequence of Figure 3 or the amino acid sequence of Figure 4.

7. Purified D type cyclin of Claim 5 which is selected from the group consistin of: cyclin D1, cyclin D2 and cyclin D3.

8. Recombinant D-type cyclin of mammalian origin of approximate molecular weight 34 kD.

9. Recombinant D-type cyclin of Claim 8 having the amino acid sequence of Figure 2, the amino acid sequence of Figure 3 or the amino acid sequence of Figure 4.

10. Isolated DNA encoding D-type cyclin of mammalian origin of approximate molecular weight 34 kD.

11. Isolated DNA of Claim 10 having the nucleic acid sequence of Figure 2, the nucleic acid sequence of Figure 3 or the nucleic acid sequence of figure 4.

12. Isolated DNA encoding a D-type cyclin protein which replaces a CLN-type protein essential for cell cycle start in budding yeast.

13. A DNA probe which hybridizes to at least a portion of a nucleic acid sequence selected from the group consisting of: the nucleic acid sequence of Figure 2, the nucleic acid sequence of Figure 3 and the nucleic acid sequence of Figure 4.

14. A DNA probe of Claim 13 which is labelled.

15. A labelled DNA probe of Claim 14 wherein the label is selected from the group consisting of: radioactive labels, fluorescent labels, enzymatic labels and binding pair members.

16. An antibody which specifically binds D-type cyclin of mammalian origin of approximate molecular weight 34 kD.

17. An antibody of Claim 16 which is a labelled monoclonal antibody.

18. A method of identifying DNA which replaces a gene essential for cell cycle start in yeast, comprising the steps of:

a) providing mutant yeast cells in which the gene essential for cell cycle start is conditionally expressed;

b) introducing into mutant yeast cells of (a) a yeast vector which contain DNA to be assessed for its ability to replace a gene essential for cell cycle start in yeast and which expresses the DNA in the mutant yeast cells; and

c) selecting transformed mutant yeast cells produced in (b) on the basis of their ability to grow under conditions under which the gene essential for cell cycle start in the mutant yeast cells provided in (a) is not expressed, wherein ability to grow under the conditions of (c) is indicative of the presence in transformed mutant yeast cells of DNA which replaces a gene essential for cell cycle start.

19. The method of Claim 18 wherein the mutant yeast cells have inactive CLN1 and CLN2 genes and an altered CLN3 gene which is conditionally expressed from a glucose-repressible promoter; the yeast vector is pADNS and screening in (c) is carried out by assessing the ability of transformed mutant yeast produced in (b) to grow in the presence of glucose.

20. The method of Claim 19 wherein the DNA which replaces a gene essential for cell cycle start in yeast is a D-type cyclin.

21. The method of Claim 20 further comprising confirming that ability to grow in the presence of glucose is not the result of reversion by affirming stability of the yeast vector in transformed mutant yeast selected in (c).

22. A method of identifying DNA encoding cyclin which replaces a gene essential for cell cycle start in yeast, comprising the steps of:

a) providing mutant yeast cells in which the CLN1 gene and the CLN2 gene are inactive and the CLN3 gene is conditionally expressed;

b) introducing into mutant yeast cells of (a) the yeast vector pADNS containing DNA to be assessed for its ability to replace the CLN3 gene, thereby producing transformed mutant yeast cells;

c) maintaining transformed mutant yeast cells produced in (b) on glucose-containing medium; and

d) selecting transformed mutant yeast cells produced in (b) on the basis of their ability to grow on glucose-containing medium.

23. The method of Claim 22 further comprising confirming the stability of the yeast vector pADNS in transformed mutant yeast cells selected in (d).

24. The method of Claim 23 wherein the cyclin which replaces a gene essential for cell cycle start in yeast is a D-type cyclin.

25. A method of detecting DNA encoding a cyclin of mammalian origin in a cell, comprising the steps of:

a) processing cells to render nucleic acid sequences present in the cells available for hybridization with complementary nucleic acid sequences;

b) combining the product of (a) with DNA encoding a D-type cyclin of mammalian origin or DNA complementary to DNA encoding a D-type cyclin of mammalian origin;

c) maintaining the product of (b) under conditions appropriate for hybridization of complementary nucleic acid sequences; and

d) detecting hybridization of complementary nucleic acid sequences,

wherein hybridization is indicative of the presence of DNA encoding a D-type cyclin of mammalian origin.

26. The method of Claim 25 wherein in (b) the product of (a) is combined with DNA selected from the group consisting of: DNA having the sequence of Figure 2; DNA complementary to the sequence of Figure 2; DNA having the sequence of Figure 3; and DNA complementary to the sequence of Figure 3.

27. The method of Claim 26 wherein the cyclin is a D-type cyclin.

28. The method of Claim 27 further comprising comparing hybridization detected in (d) with hybridization detected in appropriate control cells, wherein if hybridization detected in (d) is greater than hybridization in the control cells, it is indicative of increased levels of the DNA encoding the D-type cyclin of mammalian origin.

29. A method of detecting a D-type cyclin in a biological sample, comprising the steps of:

a) providing a biological sample to be assessed for D-type cyclin level;

b) combining the biological sample with an antibody specific for a D-type cyclin; and

c) detecting binding of the antibody of (b) with a component of the biological sample,

wherein binding is indicative of the presence of a D-type cyclin.

30. The method of Claim 29 wherein the antibody specific for a D-type cyclin is labelled.

31. A method of detecting amplification of a D-type cyclin in a biological sample, comprising the steps of:

a) providing a biological sample to be assessed for D-type cyclin level;

b) combining the biological sample with an antibody specific for a D-type cyclin;

c) determining the extent to which the antibody specific for a D-type cyclin binds to D-type cyclin in the biological sample; and

d) comparing the results of (c) with the extent to which the antibody specific for a D-type cyclin binds to D-type cyclin in an appropriate control,

wherein greater binding of the antibody to D-type cyclin in the biological sample than in the appropriate control is indicative of amplification of the D-type cyclin.

32. The method of Claim 31 wherein the antibody specific for a D-type cyclin is labelled.

33. A method of detecting in a cell an increased level of a D-type cyclin of mammalian origin, comprising the steps of:

a) processing cells to be analyzed to render nucleic acids present in the cells available for hybridization with complementary nucleic acid sequences;

b) combining the product of (a) with DNA which hybridizes with DNA encoding a D-type cyclin of mammalian origin under the conditions used;

c) maintaining the combination of (b) under conditions appropriate for hybridization of complementary nucleic acid sequences;

d) detecting hybridization of complementary nucleic acid sequences; and

e) comparing hybridization detected in (d) with hybridization in appropriate control cells,

wherein hybridization is indicative of the presence of a D-type cyclin of mammalian origin and greater

hybridization in (d) than in the control cells is indicative of increased levels of the D-type cyclin of mammalian origin.

34. A method of inhibiting cell division comprising introducing into a cell a drug which interferes with formation in the cell of the protein kinase-D type cyclin complex essential for cell cycle start.

35. The method of Claim 34 wherein the drug is selected from the group consisting of:

a) oligonucleotide sequences which bind DNA encoding D-type cyclins;

b) antibodies which specifically bind D-type cyclins;

c) agents which degrade D-type cyclins; and

d) oligopeptides.

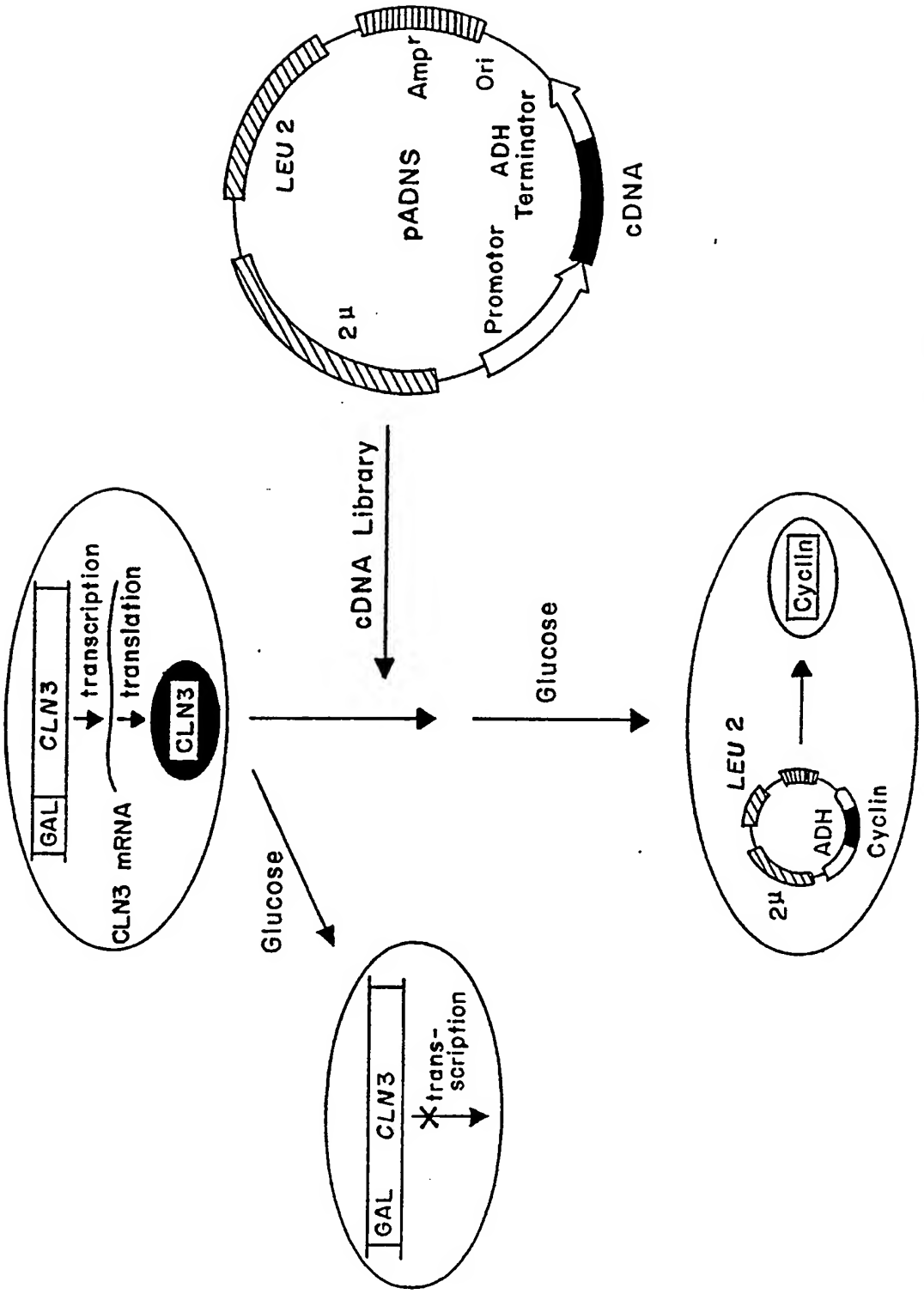
36. A method of interfering with activation in a cell of a protein kinase essential for cell cycle start, comprising introducing into the cell a drug selected from the group consisting of:

a) oligonucleotides which bind DNA encoding D-type cyclins;

b) peptides which bind the protein kinase essential for cell cycle start but do not activate it;

c) antibodies which specifically bind D-type cyclins; and

d) agents which degrade D-type cyclins.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Promotor
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FIG. 1

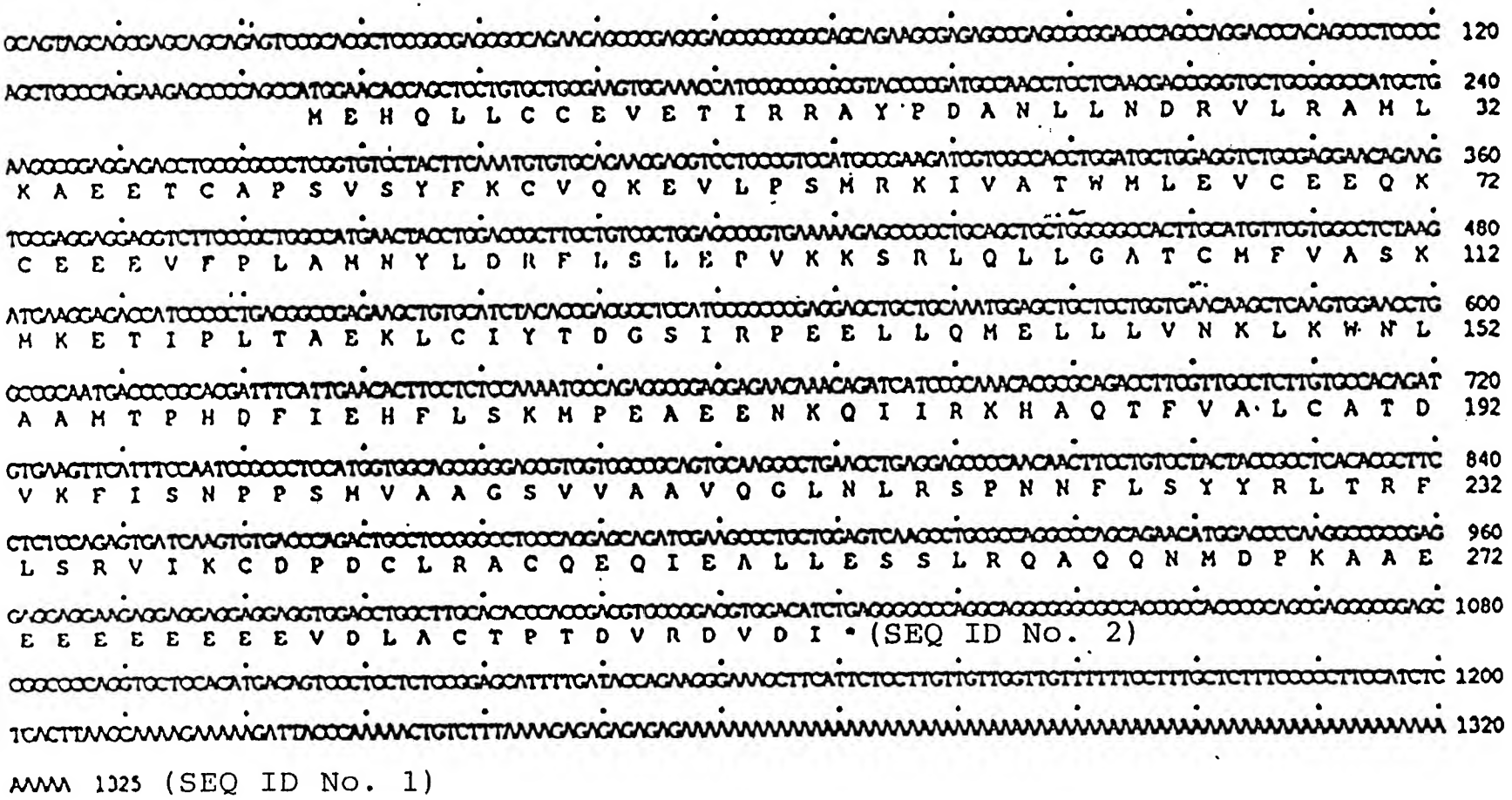

FIGURE 2

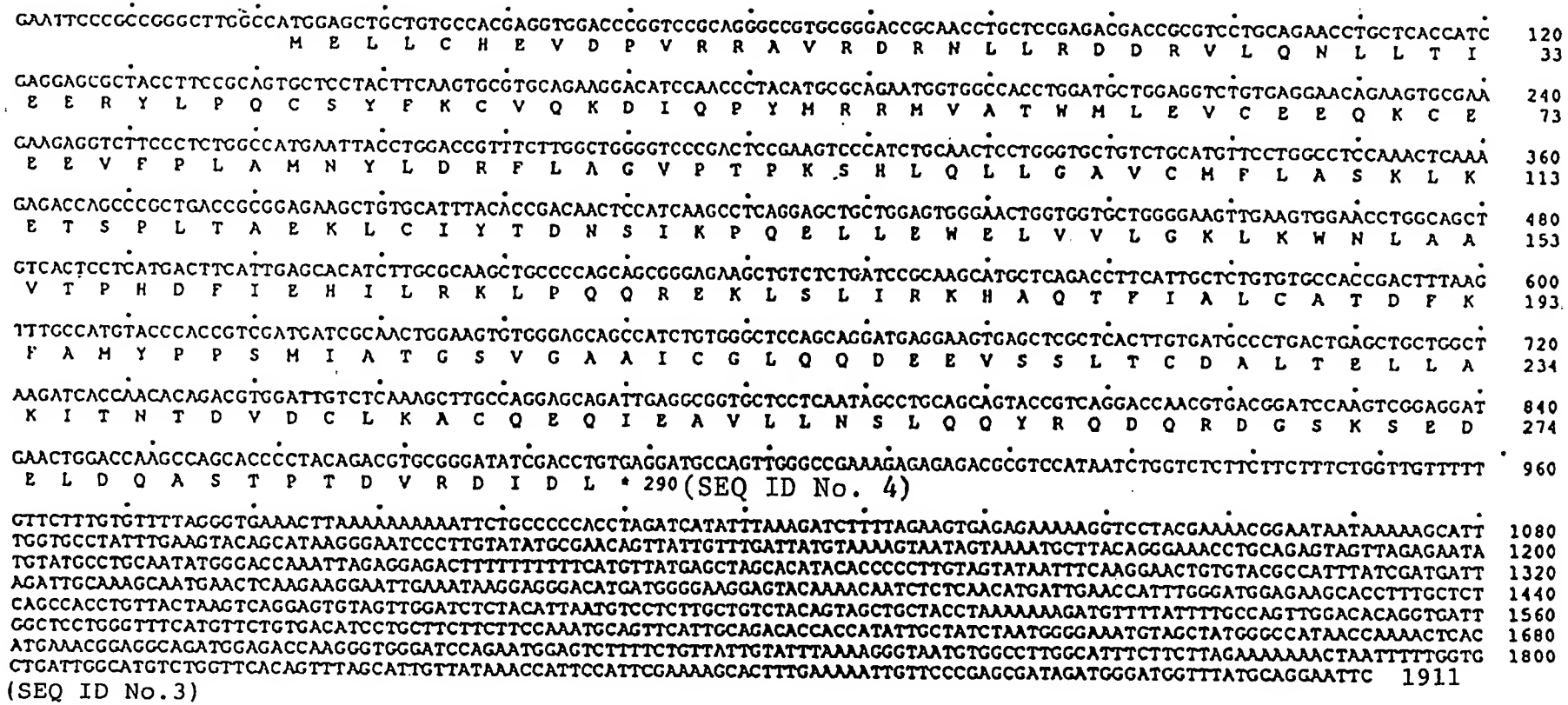

Figure 3

SUBSTITUTE SHEET

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                                                                                                  M  E  L  L  C  C  E    7

AGGCACCCGGCACGCGCCCCGGGCCGGGCCGGACCCGCGGCTGCTGGGGGACCAGCGTGTCCTGCAGAGCCTGCTCCGCCTGGAGGAGCGCTACGTACCCCGCGCCTCCTACTTCCAGTG  240
  G  T  R  H  A  P  R  A  G  P  D  P  R  L  L  G  D  Q  R  V  L  Q  S  L  L  R  L  E  E  R  Y  V  P  R  A  S  Y  F  Q  C   47

CGTGCAGCGGGAGATCAAGCCGCACATGCGGAAGATGCTGGCTTACTGGATGCTGGAGGTATGTGAGGAGCAGCGCTGTGAGGAGGAAGTCTTCCCCCTGGCCATGAACTACCTGGATCG  360
  V  Q  R  E  I  K  P  H  M  R  K  M  L  A  Y  W  M  L  E  V  C  E  E  Q  R  C  E  E  E  V  F  P  L  A  M  N  Y  L  D  R   87

CTACCTGTCTTGCGTCCCCACCCGAAAGGCGCAGTTGCAGCTCCTGGGTGCGGTCTGCATGCTGCTGGCCTCCAAGCTGCGCGAGACCACGCCCCTGACCATCGAAAAACTGTGCATCTA  480
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CACCGACCACGCTGTCTCTCCCCGCCAGTTGCGGGACTGGGAGGTGCTGGTCCTAGGGAAGCTCAAGTGGGACCTGGCTGCTGTGATTGCACATGATTTCCTGGCCTTCATTCTGCACCG  600
  T  D  H  A  V  S  P  R  Q  L  R  D  W  E  V  L  V  L  G  K  L  K  W  D  L  A  A  V  I  A  H  D  F  L  A  F  I  L  H  R  167

GCTCTCTCTGCCCCGTGACCGACAGGCCTTGGTCAAAAAGCATGCCCAGACCTTTTTGGCCCTCTGTGCTACAGATTATACCTTTGCCATGTACCCGCCATCCATGATCGCCACGGGCAG  720
  L  S  L  P  R  D  R  Q  A  L  V  K  K  H  A  Q  T  F  L  A  L  C  A  T  D  Y  T  F  A  M  Y  P  P  S  M  I  A  T  G  S  207

CATTGGGGCTGCAGTGCAAGGCCTGGGTGCCTGCTCCATGTCCGGGGATGAGCTCACAGAGCTGCTGGCAGGGATCACTGGCACTGAAGTGGACTGCCTGCGGGCCTGTCAGGAGCAGAT  840
  I  G  A  A  V  Q  G  L  G  A  C  S  M  S  G  D  E  L  T  E  L  L  A  G  I  T  G  T  E  V  D  C  L  R  A  C  Q  E  Q  I  247

CGAAGCTGCACTCAGGGAGAGCCTCAGGGAAGCCGCTCAGACCAGCTCCAGCCCAGCGCCCAAAGCCCCCCGGGGCTCCAGCAGCCAAGGGCCCAGCCAGACCAGCACTCCTACAGATGT  960
  E  A  A  L  R  E  S  L  R  E  A  A  Q  T  S  S  S  P  A  P  K  A  P  R  G  S  S  S  Q  G  P  S  Q  T  S  T  P  T  D  V  287

CACAGCCATACACCTGTAGCCCTGGAGAGGCCCTCTGGAGTGGCCACTAAGCAGAGGAGGGGCCGCTGCACCCACCTCCCTGCCTCCAGGAACCACACCACATCTAAGCCTGAAGGGGCG 1080
  T  A  I  H  L  *  292 (SEQ ID No. 6)

TCTGTTCCCCCTTCACAAAGCCCAAGGGATCTGGTCCTACCCATCCCCGCAGTGTGCACTAAGGGCCCGGCCAGCCATGTCTGCATTTCGGTGGCTAGTCAAGCTCCTCCTCCCTGCAT 1200
CTGACCAGCAGCGCCTTTCCCAACTCTAGCTGGGGGTGGGCCAGGCTGATGGGACAGAATTGGATACATACACCAGCATTCCTTTTGAACGCCCCCCCCCACCCCTGGGGGCTCTCATGT 1320
TTTCAACTGCCAAAATGCTCTAGTGCCTTCTAAAGGTGTTGTCCCTTCTAGGGTTATTGCATTTGGATTGGGGTCCCTCTAAAATTTAATGCATGATAGACACATATGAGGGGGAATAGT 1440
CTAGATGGCTCCTCTCAGTACTTTGGAGGCCCCTATGTAGTCCGTGCTGACAGCTGCTCCTAGAGGGAGGGGCCTAGGCTCAGCCAGAGAAGCTATAAATTCCTCTTTGCTTTGCTTTCT 1560
GCTCAGCTTCTCCTGTGTGATTGACAGCTTTGCTGCTGAAGGCTCATTTTAATTTATTAATTGCTTTGAGCACAACTTTAAGAGGACGTAATGGGGTCCTGGCCATCCCACAAGTGGTGG 1680
TAACCCTGGTGGTTGCTGTTTTCCTCCCTTCTGCTACTGGCAAAAGGATCTTTGTGGCCAAGGAGCTGCTATAGCCTGGGGTGGGGTCATGCCCTCCTCTCCCATTGTCCCTCTGCCCCA 1800
TCCTCCAGCAGGGAAAATGCAGCAGGGATGCCCTGGAGGTGCTGAGCCCCTGTCTAGAGAGGGAGGCAAGCCTGTTGACACAGGTCTTTCCTAAGGCTGCAAGGTTTAGGCTGGTGGCCC 1920
AGGACCATCATCCTACTGTAATAAAGATGATTGTGGGAATTC 1962 (SEQ ID No. 5)
```

Figure 4

A

```
CYCD1-Hs QLCCEVETIRRAYPDANLLNDRVLRAMLKAEETCAPSVSYFKCVQKEVLPSMRKIVATWMLEVCEEQKCEEEVFPLAMNYLDRFLSLEPVKKSRLQLLGATCMF

CYCA-Hs  SIVLEDEKPVSVNEVPDYHEDIHTYLR-EMEVKCKPKVGYMKKQP-DITNSMRAILVDWLVEVGEEYKLQNETLHLAVNYIDRFLSSMSVLRGKLQLVGTAAML
CYCA-Dm  KELPPRNDRQRFLEVVQYQMDILEYFR-ESEKKHRPKPRYMRRQK-DISHNMRSILIDWLVEVSEEYKLDTETLYLSVFYLDRFLSQMAVVRSKLQLVGTAAMY
CYCB1-Hs VNDVDAEDGADPNLCSEYVKDIYAYLR-QLEEEQAVRPKYLLGR--EVTGNMRAILIDWLVQVQMKFRLLQETMYMTVSIIDRFMQNNCVPKKMLQLVGVTAMF
CDC13-Sp WDDLDAEDWADPLMVSEYVVDIFEYLN-ELEIETMPSPTYMDRQ-KELAWKMRGILTDWLIEVHSRFRLLPETLFLAVNIIDRFLSLRVCSLNKLQLVGIAALF
CLN1-Sc  IELSNAELLTHYETIQEYHEEISQNVL-VQSSKTKPDIKLIDQQPEMNPHQTREAIVTFLYQLSVMTRVSNGIFFHSVRFYDRYCSKRVVLKDQAKLVVGTCLW
CLN3-Sc  PNLVKRELQAHHSAISEYNNDQLDHYF-RLSHTERPLYNL3NSQPQVNP-KMRFLIFDFIMYCHTRLNLSTSTLFLTFTILDKYSSRFIIKSYNYQLLSLTALW

CYCD1-Hs VASKVKETIPLTAEKLCIYTDGSIRPEELLQMELLLVNKLKWNLAAMTPHDFIEHFLSKMPEAEENKQIIRKHAQTFVALCATDVKFISNPPSMVAAGSVVAAV
         (SEQ ID #7

CYCA-Hs  LASKFEEIYPPEVAEFVYITDDTYTKKQVLRMEHLVLKVLTFDLAAPTVNQFLTQ-YFLHQQ2NCKVESLAMFLGELSLIDAD--PYLKYLPSVIAGAAFHLAL
         (SEQ ID #8)

CYCA-Dm  IAAKYEEIYPPEVGEFVFLTDDSYTKAQVLRMEQVILKILSFDLCTPTAYVFINT-YAVLCDMPEKLKYMTLYISELSLMEGE--TYLQYLPSLMSSASVALAR
         (SEQ ID #9)

CYCB1-Hs IASKYEEMYPPEIGDFAFVTDNTYTKHQIRQMEMKILRALNFGLGRPLPLHFLRR-ASKIGEVDVEQHTLAKYLMELTMLDYD---MVHFPPSQIAAGAFCLAL
         (SEQ ID #10

CDC13-Sp IASKYEEVMCPSVQNFVYMADGGYDEEEILQAERYILRVLEFNLAYPNPMNFLRR-ISKADFYDIQTRTVAKYLVEIGLLDHK---LLPYPPSQQCAAAMYLAR
         (SEQ ID #11)

CLN1-Sc  LAAKTWG25RLSELVHYCGGSDLFDESMFIQMERHILDTLNWDVYEPMINDYI     (SEQ ID #12)
CLN3-Sc  ISSKFWD3RMATLKVLQNLCCNQYSIKQFTTMEMHLFKSLDWSI2SATFDSYI     (SEQ ID #13)
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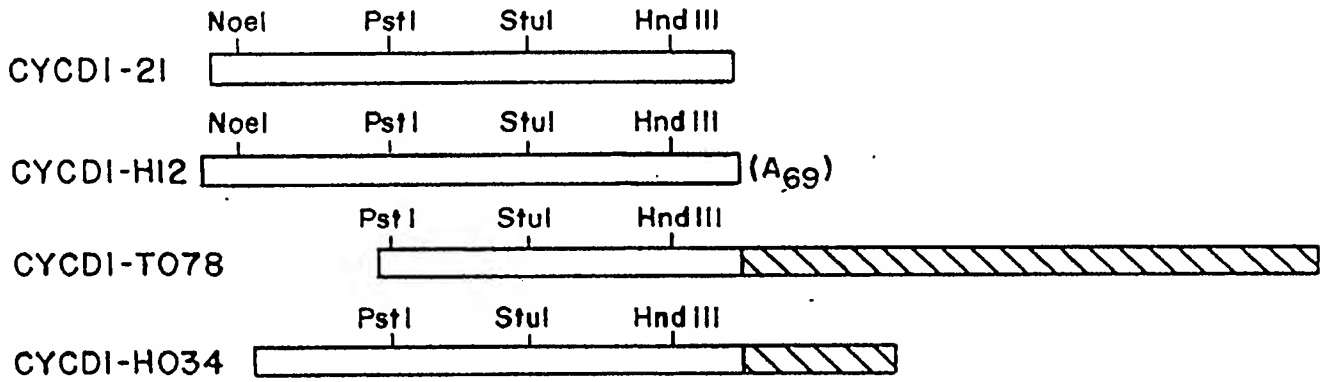
FIG. 5A

A



B CYCD1-21 ....CCCAAAAACTGTCTTT cDNA (Glioblastoma) (SEQ ID #14)
CYCD1-H12 ....CCCAAAAACTGTCTTTAAAAGAGAGAGAGAG(A69) cDNA (HeLa) (SEQ ID #15)
CYCD1-H034 ....CCCAAAAACTGTCTTTAAAAGAGAGAGAGAGAAAAAAAAAATAGTATT
CYCD1-T078 ....CCCAAAAACTGTCTTTAAAAGAGAGAGAGAGAAAAAAAAA-TAGTATT
CYCD1-G068 ....CCCAAAAACTGTCTTTAAAAGAGAGAGAGAGAAAAAAAAAATAGTATT
CYCD1-H034 TGCATAACCCTGAGCGGTGGGGGAGGAGGGTT... cDNA (HeLa) (SEQ ID #16)
CYCD1-T078 TGCATAACCCTGAGCGGTGGGGGAGGAGGGTT... cDNA (Teratocarcinoma) (SEQ ID #17)
CYCD1-G068 TGCATAACCCTGAGCGGTGGGGGAGGAGGGTT... genomic (liver) (SEQ ID #18)

C

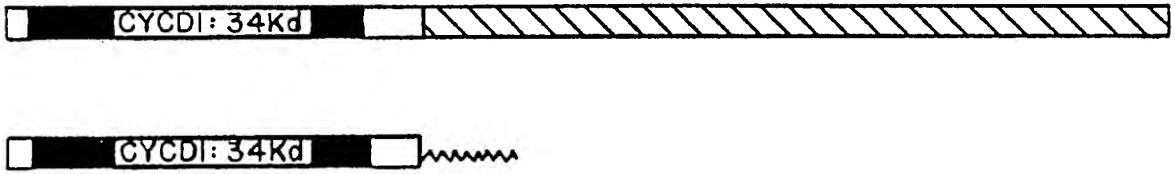


FIG. 6

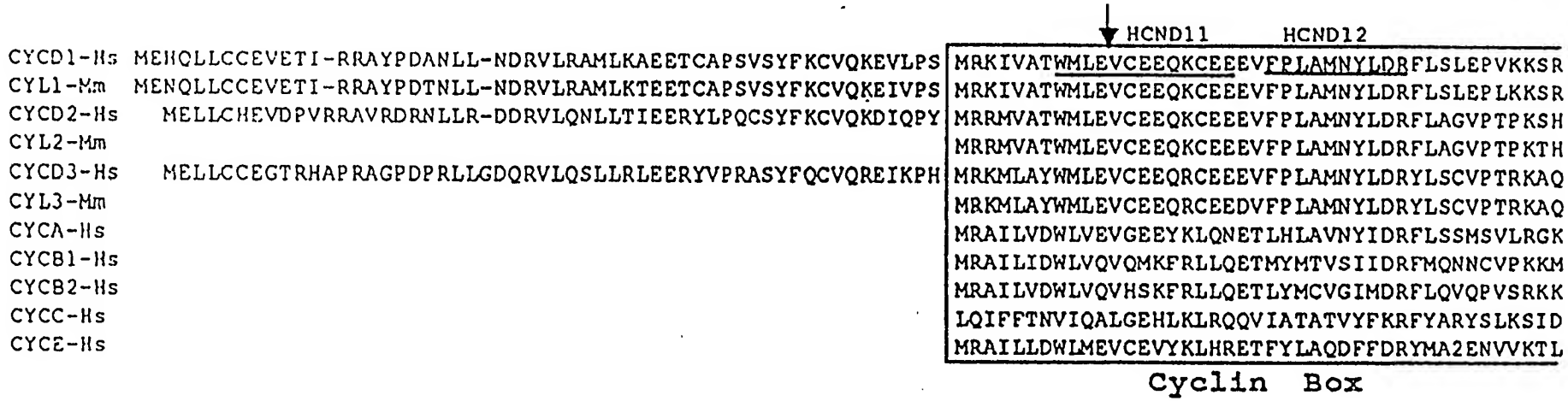


FIGURE 7 (Page 1 of 3)

SUBSTITUTE SHEET

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                                    HCND13
CYCD1-Hs LQLLGATCMFVASKMKETIPLTAEKLCIYTDGSIRPEELLQMELLLVNKLKWNLAAMTPHDFI EHFLSKMPEAEENKQIIRKHAQTFVALCATDVKFISN
CYL1-Mm  LQLLGATCMFVASKMKETIPLTAEKLCIYTDNSIRPEELLQMELLLVNKLKWNLAAMTPHDFI EHFLSKMPEAEDNKQTIRKHAQTFVALCATDVKFISN
CYCD2-Hs LQLLGAVCMFLASKLKETSPLTAEKLCIYTDNSIKPQELLEWELVVLGKLKWNLAAVTPHDFI EHILRKLPQQREKLSLIRKHAQTFIALCATDFKFAMY
CYL2-Mm  LQLLGAVCMFLASKLKETIPLTAEKLCIYTDNSVKPQELLEWELVVLGKLKWNLAAVTPHDFI EHILRKLPQQKEKLSLIRKHAQTFIALCATDFKFAMY
CYCD3-Hs LQLLGAVCMLLASKLRETTPLTIEKLCIYTDHAVSPRQLRDWEVLVLGKLKWDLAAVIAHDFL AFILHRLSLPRDRQALVKKHAQTFLALCATDYTFAMY
CYL3-Mm  LQLLGTVCILLASKLRETTPLTIEKLCIYTDQAVAPWQLREWEVLVLGKLKWDLAAVIAHDFL ALILHRLSLPSDRQALVKKHAQTFLALCATDYTFAMY
CYCA-Hs  LQLVGTAAMLLASKFEEIYPPEVAEFVYITDDTYTKKQVLRMEHLVLKVLTFDLAAPTVNQFL    (SEQ ID #25)
CYCB1-Hs LQLVGVTAMFIASKYEEMYPPEIGDFAFVTDNTYTKHQIRQMEMKILRALNFGLGRPLPLHFL    (SEQ ID #26)
CYCB2-Hs LQLVGITALLLASKYEEMFSPNIEDFVYITDNAYTSSQIREMETLILKELKFELGRPLPLHFL    (SEQ ID #27)
CYCC-Hs  PVLMAPTCVFLASKVEE16LKTRFSYAFPKEFPYRMNHILECEFYLLELMDCCLIVYHPYRPL    (SEQ ID #28)
CYCE-Hs  LQLIGISSLFIAAKLEEIYPPKLHQFAYVTDGACSGDEILTMELMIMKALKWRLSPLTIVSWL    (SEQ ID #29)
                                    Cyclin Box
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FIGURE 7 (Page 2 of 3)

CYCD1-Hs PPSMVAAGSVVAAVQGLNLRSPNNFLSYYRLTRFLSRVIKCDPDCLRACQEQIEALLESSLRQAQQNMDPKA-AEEEEEEEEEVDLACTPTDVRDVDI*
(SEQ ID #19)

CYL1-Mm PPSMVAAGSMVAAMQGLNLGSPNNFLSRYRTTHFLSRVIKCDPDCLRACQEQIEALLESSLRQAQQNVDPKA-TEEEGEVEEEAGLACTPTDVRDVDI*
(SEQ ID #20)

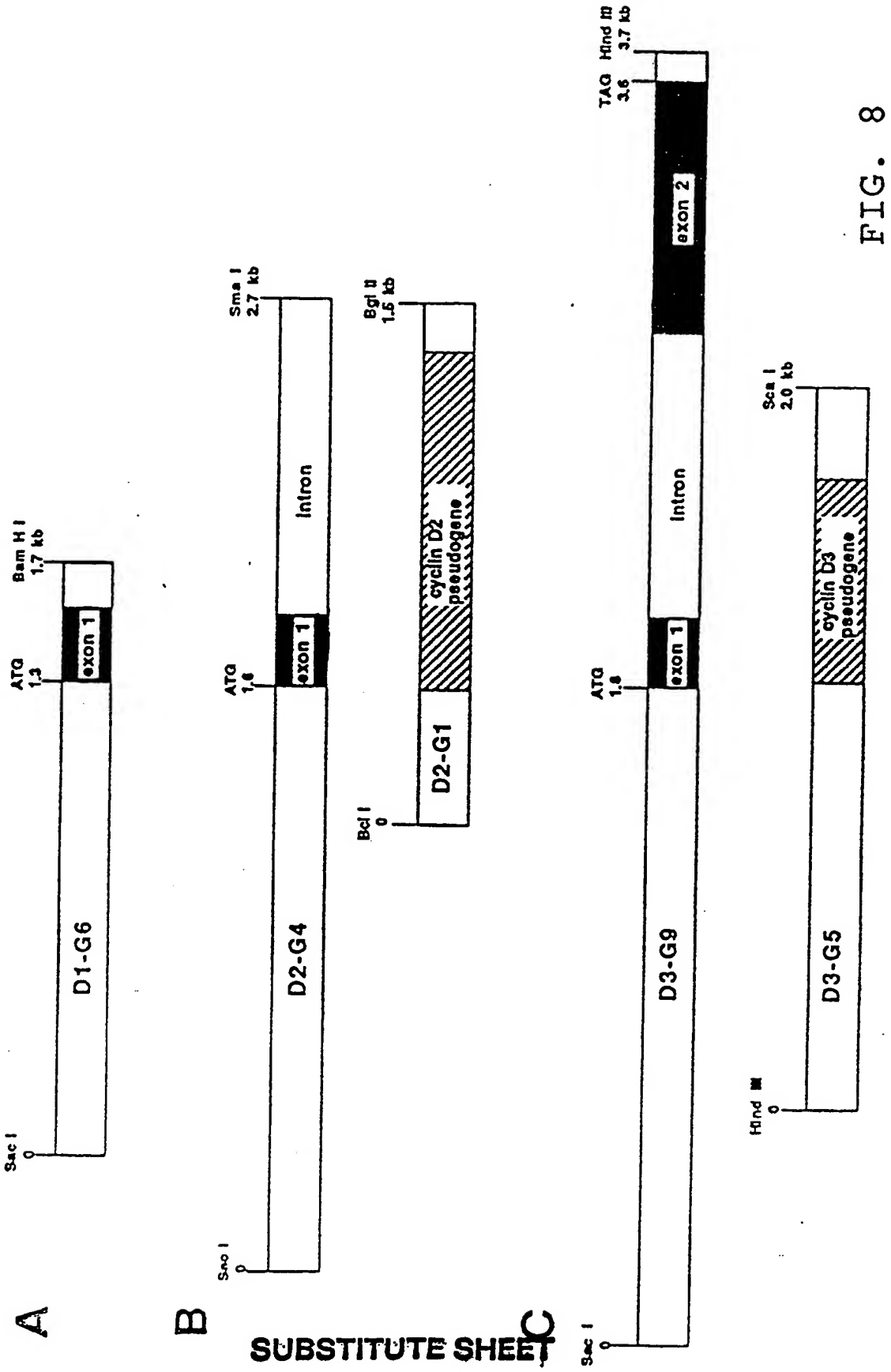
CYCD2-Hs PPSMIATGSVGAAICGLQQDEEVSSLTCDALTELLAKITNTDVDCLKACQEQIEAVLLNSLQQYRQDQRD------GSKSEDELDQASTPTDVRDIDL*
(SEQ ID #21)

CYL2-Mm PPSMIATGSVGAAICGLQQDDEVNTLTCDALTELLAKITHTDVDCLKACQEQIEALLLNSLQQFRQEQHNA-----GSKSVEDPDQATTPTDVRDVDL*
(SEQ ID #22)

CYCD3-Hs PPSMIATGSIGAAVQGLGACS----MSGDELTELLAGITGTEVDCLRACQEQIEAALRESLREAAQTSSSPAPKAPRGSSSQGPSQTSTPTDVTAIHL*
(SEQ ID #23)

CYL3-Mm PPSMIATGSIGAAVIGLGACS----MSADELTELLAGITGTEVDCLRACQEQIEAALRESLREAAQTAPSPVPKAPRGSSSQGPSQTSTPTDVTAIHL*
(SEQ ID #24)

FIGURE 7 (Page 3 of 3)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pseudogene
D2-G1
Bcl I
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C
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3.6
Hind III
3.7 kb
exon 2
Intron
ATG
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exon 1
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Sca I
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cyclin D3
pseudogene
D3-G5
Hind III
0

FIG. 8

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TGATCAAGTTGACACTCAATATTAACCCTCATAGACTGTGATCCCTATGTTGCTGCCTTCCCTCGTTTCTATTGCTCTTTGGCCCCAACCCAAATAAGGTTCCTTGGGACACACTAAAGA  120
AGGAGGTGGAGTTCGAAGGGGAGGAGAGATGTGAGCGAGGCAGGCAGGGAAGCTCTGCTCGCCCACTGCCCAATCCTCACCTCTCTTCTCCTCCACCTTCTGTCTCTGCCCTCACCTCTC  240
CTCTGAAAACCCCCTATTGAGCCAAAGGAAGGAGATGAGGGGAATGCTTTTGCCTTCCCCCTCCAAAACAAAAACAAAAACAAACACACTTTTCCAGTCCAGAGAAAGCAGGGGAGTGAG  360
GGGTCACAGAGCTGGCCATGCAGCTGCTGGGCTGTGAGGTAGACCCGGTCCTCAGAGCCACGAGGGACTGCAACCTACTCCAAGTTGACCGTGTCCTGAAGAACCTGCTTGCTATCAAGA  480
                    M  Q  L  L  G  C  E  V  D  P  V  L  R  A  T  R  D  C  N  L  L  Q  V  D  R  V  L  K  N  L  L  A  I  K

AGCGCTACCTTCAGTAATGCTCCTACTTCAAGTGTGTGCAGAAGGCCATCCAGCCGTACATGCACAGGATGGTGCCACTTCTGATGGTGGCCATTTGATTGGTGCCACTTCTGATGGTGG  600
K  R  Y  L  Q  *  C  S  Y  F  K  C  V  Q  K  A  I  Q  P  Y  M  H  R  M  V  P  L  L  M  V  [        Insertion

CCAACATGATTGAACCATTTGGGATGGAAAAGCACCTTTACTCTCAGCCACCTGTTAACTAATGCTGGAGGTCTGTGAGGAACAGAAGTGTGAAGAAAAGGTTTTCCCTCTGGCCACGAT  720
                                                                                  ]  M  L  E  V  C  E  E  Q  K  C  E  E  K  V  F  P  L  A  T  I
            Insertion

GTACCTGGACTGTTTCTTCGCCAGGATCCCAACTTCAAAGTCCCATCTGCAACTCCTGGGTGCTGTCTGCATGTTCCTGGCCTCCAGGCTCAAAGAGTCCAGCCCACTGACTGCCAAAAA  840
Y  L  D  C  F  F  A  R  I  P  T  S  K  S  H  L  Q  L  L  G  A  V  C  M  F  L  A  S  R  L  K  E  S  S  P  L  T  A  K  K

CCTGTGCATTTATACCGACAACTCCATCAAGCCTCAGGAGCTGCTGGAGTGGGAACTGGTGGTGTTGGGAAAGTTGAAGTGGAACCTGGCAGCTGTCACGCCTCATGACTTCATTTAGTA  960
L  C  I  Y  T  D  N  S  I  K  P  Q  E  L  L  E  W  E  L  V  V  L  G  K  L  K  W  N  L  A  A  V  T  P  H  D  F  I  *  Y

CATCTTGCACAAGCTGCCCCAGCAGCGGGAGAAGCTGTCTCCAATCTGCAAGCAAGTCCAGAACTTCAATGCTCTGTATGCAATGTACCCGCCATCAATGGTTGCAACTGGAAGTGTAGG 1080
I  L  H  K  L  P  Q  Q  R  E  K  L  S  [        deletion                    ]  A  M  Y  P  P  S  M  V  A  T  G  S  V  G

AGCAGCTATCTGTGGACTTCAGCAACATGAGGAAGTGAGCTCACTCCCTTGCAATGCCCTGACTGAGCTGCTGGCAAAGATCACCAACACAGATGTGGATTGTCTCAAAAGCCAACCGGG 1200
A  A  I  C  G  L  Q  Q  H  E  E  V  S  S  L  P  C  N  A  L  T  E  L  L  A  K  I  T  N  T  D  V  D  C  L  K  \  A  N  R

AGCATATTGAGGTGGTCTTCCTCAACAGCCTGCAGCAGTGCCATCAGGACCAGCAGGACAGATCCAAGTCAGAGGATGAACTGGGCCAAGCAGCACCCCTATAGACCTGTGAGATATCGA 1320
E  H  I  E  V  V  F  L  N  S  L  Q  Q  C  H  Q  D  Q  Q  D  R  S  K  S  E  D  E  L  G  Q  A\S  T  P  I  D  L  *  D  I  D

CCTGTGAGGATGGCAGTCCAGCTGAGAGGCGCATTCATAATCTGCTGTCTCCTTCTTTCTGGTTATGTTTTGTTCTTTGTATCTTAGGGCGAAACTTAAAAAAAAAAAACCTCTGCCCCCA 1440
  L  *  (SEQ ID No. 31)

CATAGTTCGTGTTTAAAGATCT  1462 (SEQ ID No. 30)
```

FIG. 9

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AAGCTTCCAGATTAGAAAAGAAAAAATAAAACTATCTTTATTTGCAGATGACATGATCGGTCCATTCTCATGCTGCTTATAAAGACATACCCAAGACTGGATAATTTATAAAGGAAAGAG  120
GTTTGGCTCACAGTTCCCCATGGGTGGAGAGGCCTCACAATCATGGCGAAAGAGCAAGGAGCATCTCACATGGCAGCAGGCAAGAAAAGAATGAGAGCCACGCCAGAGGGAAACCCCTTA  240
TAAAATCATCAGATCTCGAGAGACTTATTCACTGTCAGGAGAACAGTATGGAGGAAACGCCCTTATGATTCAATTATCTCGCACTGTGTTCCTCCCACAACACATGGGAATTATGGGAGC  360
TACAATTCAAGATGAGATTTGGGTGGAGACACAGCCAAACCATATCAATCTTTTTTTTCTTATTCTTTTTTTTTTTTTTTTTTTTTTTTTTTGAGATGGAGTCCCACTCTGTTATCTAGGCTGG  480
AGTGCAGTGGTGTGTGATCTTGGCTCACTGCAACCTCAGCCTCCCAGGTTCAAGCGATTCTCCTGCCTCAGACTCCTGAATAGCTGAAATTACAGGCACCTGCCACTACGCCTGGCAAAT  600
ATTTTTTGTTTGTTTGTTTGTTTGTTTGTTTTGAGACAGAGTCTCTCTCTGTCGCCCAGGCTGGAGTGCAGTGGGCGCGATCTCAGCTCACTGCAAACTCTGCTCCCGGGTTCAAG  720
CCATTCTCCTGCCTCAGCTCCCAAGTAGCTGGGACTACAGGCGCCCACCACCACCATGCCAGGCTAATTTTTTGTATTTTTAGTAGAGACAGGGTTTCACCGTGTTAGCCAGGATGGTCT  840
CAATCTCCTGACCTCGTGATCCGCCCACCTCGGCCTCCCAAAGTGCTGGGATTACAGGCGTGAGCCACTATGCCCAACCGTATCAATCTTGTATATAGAAAAACCTAAGGAATCTACAAA  960
AAAACCCTATTATAACTAATATAATAATAATCTGCAAAGTTGTAGACTATGAGATCAATATACAAAAATTAACTCAATTTCTTTACATGTACAATGAATAACCCCAAAACAAAACTGGGA 1080
ATATAATTCTATTTTTAATAGTATCACAAAGAATGACAATACTTAGAAACAAATGATGGGCGCTAGCTTGCACTCCCGCCCTGCCTGTGCGCTGCCCGAGTGTGGAGCTGCTATGCTGCG 1200
                                                    * W A L A C T P A L P V R C P S V E L L C C

AAGGCTCGAGGACCCGCAGACGCCAGGGGATCAGCGCGTCCTGCAGAGCTTGCTCCCCTTGGAGTAGCGCTGCGTGCACTGCGCCTACTTCCAGTGCGTGCAAAGGGAGAGCAAGCCGCA 1320
E G S R\D P Q T P G D Q R V L Q S L L P L E * R C V H C A Y P Q C V Q R E S K P H

CCATGCGGAAGATGCTGGTTTACTGGATGCTGGAGGTGTGTGAGGAGCAGTGCTGTGAGGAGGAGCAGTGCTGTAAGGAGGAAGTCTTTCCCCTGGCCATGAACCACCTGCATGCTACCTG 1440
  M R K M L V Y W M L E V C E E Q C C E E E Q C C K E E V F P L A M N H L H A T C

TCCTACGTCCCCACCCACCCGAAAGGCACAGTTGCAGCTCTTGGTTGCGGTCTCCATGCGGCTGGCCTCCAAGCTGCGTAAGACTGGGCCCATGACCATTGAGAAAATGTGCATCTACAC 1560
  P T S P P T R K A Q L Q L L V A V S M R L A S K L R K T G P M T I E K M C I Y T

CGACCACGCTGTCTCTCCCTGCCAGTTGCGGGACTGGGAGGTGATGGTCCTGGGGAAGCTCAAATGGGACCTGGCCGCTGTGATTGCTCATGACTTCTTGGCCCTCATTCTGCACCGACC 1680
  D H A V S P C Q L R D W E V M V L G K L K W D L A A V I A H D F L A L I L H R \

GACAGGCCTTGGTCAAAAAGCATGCCCAGATCTTTTTGGCTGTCTGTGCTACAGATTACACCTTTGCCATGTACCCACCATCCAGTTGTGAAAACAACCCAAATGCCTGTTAACTGATGA 1800
R Q A L V K K H A Q I F L A V C A T D Y T F A M Y P P S S C E N N P N A C *
(SEQ ID No. 33)
ACAGATAACCATATGTGATATATATCAATACAATGGAATATGGCCTGGCATGCTGGCTTACGCTGTAATCCTGCACTTTGGGAGGCCAAAGTGGAGGATCACTTGAGCCGAGGAGTTCAA 1920
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(SEQ ID No. 32)
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FIG. 10

GAGCTCGATCAGTACACTCGTTTGTTTAATTGATAATTGTCCTGAATTATGCCGGCTCCT
GCAGCCCCCTCACGCTCACGAATTCAGTCCCAGGGCAAATTCTAAAGGTGAAGGGACGTC
TACACCCCCAACAAAACCAATTAGGAACCTTCGGTGGGTCTTGTCCCAGGCAGAGGGGAC
TAATATTTCCAGCAATTTAATTTCTTTTTTAATTAAAAAAAATGAGTCAGAATGGAGATC
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TTCTTGCAATTTACACGTGTTAATGAAAATGAAAGAAGATGCAGTCGCTGAGATTCTTTG
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CTTGGATATGGGGTGTCGCCGCGCCCCAGTCACCCCTTCTCGTGGTCTCCCCAGGCTGCG
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TCCCGGGACCCACTCGAGGCGGACGGGCCCCCTGCACCCCTCTCGGCGGGGAGAAAGGCT
GCAGCGGGGCGATTTGCATTTCTATGAAAACCGGACTACAGGGGCAACTGCCCGCAGGGC
AGCGCGGCGCCTCAGGGATGGCTTTTCGTCTGCCCCTCGCTGCTCCCGGCGTTCTGCCCG
CGCCCCCTCCCCCTGCGCCCGCCCCCGCCCCCCTCCCGCTCCCATTCTCTGCCGGGCTTT
GATCTTTGCTTAACAACAGTAACGTCACACGGACTACAGGGGAGTTTTGTTGAAGTTGCA
AAGTCCTGGAGCCTCCAGAGGGCTGTCGGCGCAGTAGCAGCGAGCAGCAGAGTCCGCACG
CTCCGGCGAGGGGCAGAAGAGCGCGAGGGAGCGCGGGGCAGCAGAAGCGAGAGCCGAGCG
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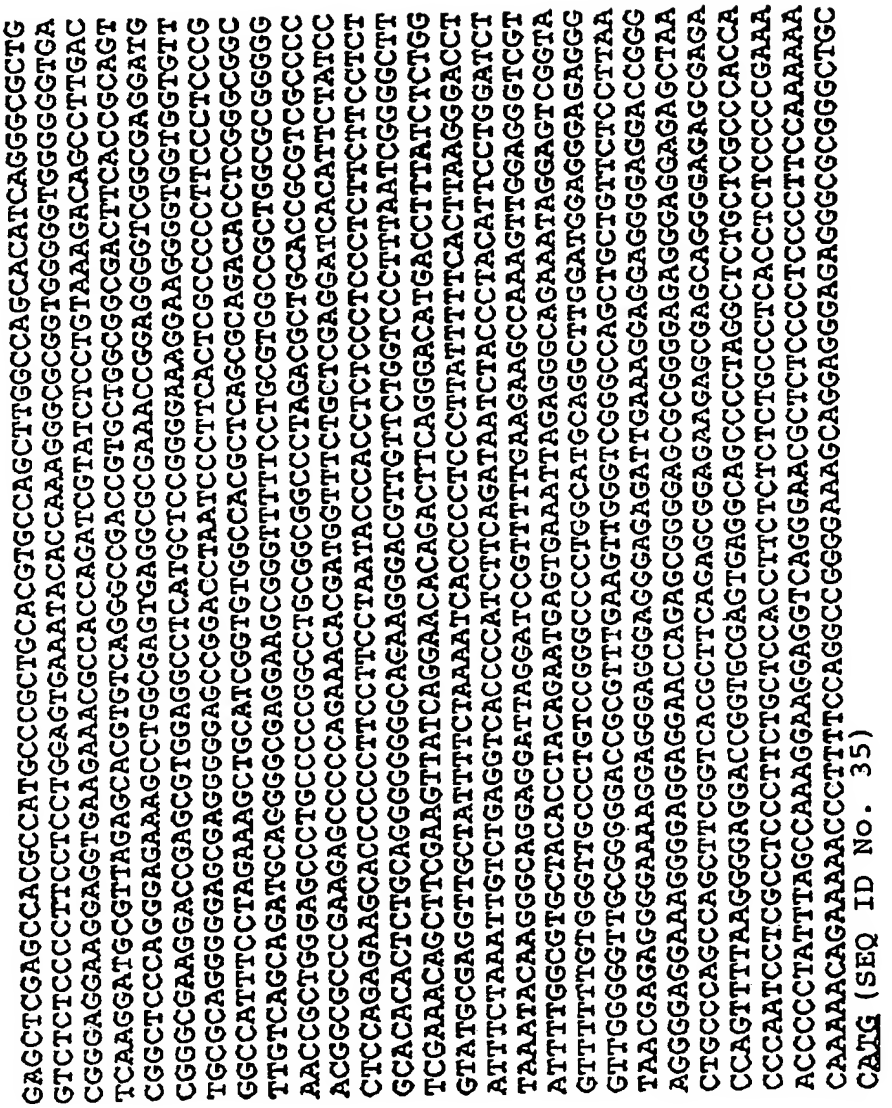
FIG. 11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TAACGAGAGGGGGAAAAGGAGGGAGGGAGATTGAAAGGAGGGGGAGGAGGACCGGG
AGGGGAGGAAAGGGGAGGAGGAACCAGAGCGGGGAGCGCGGGGAGAGGAGGAGAGCTAA
CTGCCCAGCCAGCTTCGGTCACGCTTCAGAGCGGAGAAGAGCGAGCAGGGGAGAGCGAGA
CCAGTTTTAAGGGGAGGAGACCGGTGCGAGTGAGGCAGCCCCCTAGGCTCTGCTCGCCCACCA
CCCAATCCTCGCCTCCCTTCTGCTCCACCTTCTCTCTGCCCTCACCTCTCCCCCGAAA
ACCCCCTATTTAGCCAAAGGAAGGAGGTCAGGGAACGCTCTCCCCCTCCCCTTCCAAAAAA
CAAAAACAGAAAAACCCTTTCCAGGCCGGGGAAAGCAGGAGGGAGAGGGCGCGGGCTGC
CATG (SEQ ID No. 35)

FIG. 12

GAGCTCCCGTCCCCATACTACAGGTTCACATCCAGCTTTCAGGACTAGTCAGTCTATGTG
GCCCTCCCTCAATTAATAAATCAGCAACTAATTTGCCAGGTGCGGTGGTTTGTGCCTGTA
ATCCCAGCACTTTAGGAAGCTGAGGCAGGCAGATCACTTGAGGTCAGGAGTTCGAGACCA
GCCTGGCCAACATGGTGAAATCCCGTATCTACTGAAAATACAAAAATTAGCCGGGCATGG
TGGTATGCACCCGTAATCCCAGCTACTCAGGAAGCTGAGGCAGGAGAATCACTTGAAACC
GGGAGGCAGAGGTTGCAGTAAGCTGCACTCCAGCCTGGTGACAAGAGCAAAACTTTGTGT
CAAAAAAACAAAGAAAACCAAAAAACAAAGGAAAACACAAAAAACCCTTCTATTTGTTAA
AAAAAAAAAAATCCACCGTGAACCAAAAATTAGTAAAAACAATGAACTAAAATTTTGTTT
TTGCAAAATGTATGATAACAAAATGTTAAGGAAGGTCATGTGCCGTTATGGTTCACTGCA
GCCTTGAACTCCTGGGCTCAAGCGATCCTCCTGCTTCGGTCTCCCTAGTAGCTGGGACTA
CAGGCTTGTGCCACCGCACCCAGCTTATTTTTTTTTTTATTTTTGTAGAGATAGGAGT
CTTGCTTTGTTGTCCAGGCTGGTCTTCAACTCCTAGCTTCCAGTGATCCTCCTGCCTCAG
CCTCCCAAGTGCTGGGCCTGATGGGACATTTTTATACATAGTGCCATGTACCTATAAATG
AGAAGTTTTAAAAATACTGATTTTAAAAATTAATTTATGTCAAGAATTTTTATACCAAAG
TTAAAAAACCAAACCGAAAATATGAAAAGGGTTAATATCTTTGAGAGGTGATGAGAACTT
ATAAGTCAATAAGAGAAAACAAACATCCCTATAAATGAATAAGCTAAGGACATGAATGGG
TAATGTACATAAGAAATGTAAATGTCTAGTAATATGCCAAAATAGATTTATTATTACTAA
TAAGCCACTTTCACTCTCTAGTTGGCAGAGTTGTTTTGAAAAATAGATATGTAATGATGG
TGGAAAAGATTGGTTTAACTATTCAGCAGGAAAATTTGGCAATTAGAAGTGTATCAAAAG
CCTTAGAATGTTTCATAACCTTAGATTGGGAAATTCCACTTCTAGAAATTAATTCACTTC
TAGAAATAATCATGAGTGTGCACAAAGATATTACCACAAAAATATTTTACAGTATTATGT
CTAATAGAGAAGAACTAGAAATAATTTAAATTTCCACCAATACAGGTTTGCCAAAATACA
TTTTGTACATTCACCTAATGGTATATTATGTCCCTATTACAAATTACGTCCTAGAATATT
TAATAGCATGGAAAAGTGTTAACAGTATTTTTTTAATGAAAAAAGCTTACAAAACAGTTT
GTGATGATTCCATTTAAAATGTGTGTTTATTCATAGAACAAAGATTAGAAAAATAAACAT
TGATATATTAAAGGGTTATTTCATGGCAAATTGCAAATGATTATTTCCTTTTTTTGTGGC
TTATTTGTATTTTTGAAGTTTTCTACAATGTAAAAGAATATTTTATGATATGAAAACTAC
AATACAATTTATAATATAAGAAAGAATAATTCGGCCGGGAACGGTGGCTCACGCCTGTAA
TCCCAGCACTTTTGGAGGCCGAGACCGGCGGATCACGAGGTCAGGGGTTCAAGACTAGCC
TGGCCAACATAGTGAAACCCCATCTCTACGAAAAATACAAAAATTAGTCAGGCATGGTGG
TGCGTGCCTGTAGTCCCAGCTACTCGGGAATTGCTTGAACCCGGGAGGTGGAGGTTGCAG
TGAGCCCAGATCGCACCACTGCACTCCAGCTTGAGCAACAGAGTAGACTTCGTCTCAAAA
AAAAAAAAAAAAAAAAAAAGAATAATTAACAGAAAATGGTTAGACACTTCCTTAGTGTCT
CCTAAGTCAGGAGGACCCCAGTAGGGCAGGGATCCTCATGGCCTCCTCCCATTTGGAGCA
TTATTGGAGGTCTTTTTCGGCCTCTTCGTCAAGTGGAATCTAGCTTCCGGTAAAACTACA
AAGTAACCAAAAGTTTGGGAGGTGGAAGAAATGCAACCGGTAGATCTCACAGAGTCTGTG
CAAGAAACTGATTCAATGAGAATCTAGTTTCTCCGTCCACAGTTTCTCCAAACAGAAACT
AAGGCCGACTTTAGGGGCTTGTCCAAACCTAGGCAAGCAACTTAACAAGGTGAGGCCATG
ACTCCATGGCCTTTCCGTTCTGTTATATGCTGACTTAGACTAAAGCTCTCATACTTTAAA
GTGCACAGAAATCTAGTTAAAATGCAGATTCTGATTCAGGTTAGGGGTGGGCCTGAGAGT
CTGCATTTCTAACCAGCTCCCAGGCGATGACCACGCACGGGACAGGTCTGGGATCACAGT
TTAACTAGCAATGGTGTAGAACACAGAATCTGCAGCAAGAAGGCCAGCTTCCCAATCCTA
GCTCTGCCACGGACCAACTGAATGACAGTTGCCTCGGTTTCCGAGTTTTCGTGAAGATGT
AGTGAGTCATTACATCGTGAGGCTTTCGAGCAGCGTTCACTAAGAACTAGCTCTGACATT
ATTTATCGCATTCCTTAGAGCAAGCAGCCGGTGAAGTAGGGTTTGACGAATGAATAAGTG
AATGAATGACCTTTGGAGAAAAATTGTTTCCTGGGTGACTAGAGTCCGAGAAGCAAAATG
GGAGGGCCCGTGGTGGGTAGGAGGCCCACCTCCTAGAAAGTTCTCTGCACCCGGTGGTCC
AGAGGGCCTGGAGTGCCGGAAGCCGGCCGCGTTGCGCTCACGGCCCAATGGGGCCGCGGG
AGGGAGGGGAGAGCGCTCAGCCAACCCTTTCCGTTCCGGGCGCCGCAGCCCCGCCCCTCG
GAGCGTTGCGACGTCCGAGCATTCCACGGTTGCTACATCGTCGCGAGGGGGGGCGCCTGT
CAGGGAAGCGGCGCGCGCGGGCGGCGGGCGGGCTGGGGATCCGCCGCGCAGTGCCAGC
GCCAGCGCCAGACCCGCGCCCCGCGCTCTCCGGCCCGTCGCCTGTCTTGGGACTCGCGAG
CCCGCACTCCCGCCCTGCCTGTTCGCTGCCCGAGTATG (SEQ ID No. 36)

FIG. 13